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(71) Applicant *(for all designated States except US)*: INNOGENET-ICS N.V. [BE/BE]; Industriepark Zwijnaarde 7, Box 4, B-9052 Ghent (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BUYSE, Marie-Ange [BE/BE]; Burg. E. Ronsestraat 23, B-9820 Merelbeke (BE). SABLON, Erwin [BE/BE]; Robbroeklaan IA, B-1785 Merchtem (BE).

(74) Agent: DE CLERCQ, Ann; Innogenetics N.V., Industriepark Zwijnaarde 7, Box 4, B-9052 Ghent (BE).

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(54) Title: INTERFERON-GAMMA-BINDING MOLECULES FOR TREATING SEPTIC SHOCK, CACHEXIA, IMMUNE DISEASES AND SKIN DISORDERS

(57) Abstract

The present invention concerns molecules which bind and neutralize the cytokine interferon-gamma. More specifically, the present invention relates to sheep-derived antibodies and engineered antibody constructs, such as humanized single-chain Fv fragments, chimeric antibodies, diabodies, triabodies, tetravalent antibodies, peptabodies and hexabodies which can be used to treat diseases wherein interferon-gamma activity is pathogenic. Examples of such diseases are: septic shock, cachexia, multiple sclerosis and psoriasis.

INTERFERON-gamma-BINDING MOLECULES FOR TREATING SEPTIC SHOCK, CACHEXIA, IMMUNE DISEASES AND SKIN DISORDERS.

FIELD OF THE INVENTION

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The present invention concerns molecules which bind and neutralize the cytokine interferon-gamma. More specifically, the present invention relates to sheep-derived antibodies and engineered antibody constructs, such as humanized single-chain Fv fragments, chimeric antibodies, diabodies, triabodies, tetravalent antibodies and peptabodies which can be used to

10   treat diseases wherein interferon-gamma activity is pathogenic. Examples of such diseases are: septic shock, cachexia, multiple sclerosis and psoriasis.

BACKGROUND OF THE INVENTION

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Interferon-gamma (IFNγ) is a member of the interferon family of immunomodulatory proteins and is produced by activated T helper type-1 cells (Th1 cells) and natural killer cells (NK cells). Apart from its potent antiviral activity, IFNγ is known to be involved in a variety of immune functions (for a review, see Billiau, 1996) and inflammatory responses. Indeed, IFNγ

20   is the primary inducer of the expression of the major histocompatibility complex (MHC) class-II molecules (Steinman et al., 1980) by macrophages and other cell types and stimulates the production of inflammatory mediators such as tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1) and nitric oxide (NO) (Lorsbach et al., 1993). In this respect, IFNγ is shown to be important in the macrophage-mediated defence to various bacterial pathogens. Furthermore,

25   IFNγ is also shown to be a potent inducer of the expression of adhesion molecules, such as the intercellular adhesion molecule-1 (ICAM-1, Dustin et al., 1988), and of important costimulators such as the B7 molecules on professional antigen presenting cells (Freedman et al., 1991). Moreover, IFNγ induces macrophages to become tumoricidal (Pace et al., 1983) and provokes Ig isotype switching (Snapper and Paul, 1987).

30   The anti-viral, tumoricidal, inflammatory- and immunomodulatory activity of IFNγ clearly has beneficial effects in a number of clinical conditions. However, there are a number of

clinical situations in which IFNγ-activity has deleterious effects. These include cancer cachexia (Denz et al., 1993; Iwagaki et al., 1995), septic shock (Doherty et al., 1992), skin disorders such as psoriasis and bullous dermatoses (Van den Oord et al., 1995), allograft rejection (Landolfo et al., 1985; Gorczynski, 1995), chronic inflammations such as ulcerative colitis and Crohn's

5   disease (WO 94/14467 to Ashkenazi & Ward), and autoimmune diseases such as multiple sclerosis (MS, Panitch et al., 1986), experimental lupus (Ozmen et al., 1995), arthritis (Jacob et al., 1989; Boissier et al., 1995) and autoimmune encephalomyelitis (Waisman et al., 1996).

Cachexia is a phenomenon often seen in cancer patients and is associated with losses of lean body mass, and altered carbohydrate and lipid metabolism. This so called 'chronic wasting

10   syndrome' is often the immediate cause of death. In recent years, interest has focused on the role of proinflammatory cytokines in cancer related cachexia. Current data support the concept that cachexia is linked to the presence of certain cytokines among which IFNγ seems to play a central role. Denz et al. (1993) reported that increased neopterin and decreased tryptophan concentrations - which are closely related to IFNγ-activity - are detected in cachectic patients

15   suffering from hematological disorders. Neopterin is synthesized and secreted by monocytes/macrophages upon stimulation by IFNγ from activated T cells. Tryptophan is an indispensable amino acid which can be catabolized by indoleamine 2,3-dioxygenase, an enzyme induced by IFN's, and which absence initiates mechanisms responsible for cachexia (Brown et al., 1991). The correlation between high neopterin levels, decreased tryptophan levels and weight

20   loss was confirmed by Iwagaki et al. (1995). In experimental models, cancer-induced cachexia can be altered by the administration of IFNγ neutralizing antibodies (Matthys et al., 1991; Langstein et al., 1991)

Septic shock is the result of a severe bacterial infection, and remains a common cause of death among critically ill, hospitalized patients despite improvements in supportive care (Bone

25   et al., 1992). Although septic shock may be associated with gram-positive infections, attention has focused on the more common pathogenesis of gram-negative sepsis and the toxic role of endotoxin (= lipopolysaccharide or LPS), a component of the outer membrane of gram-negative and some gram-positive bacteria. Many of the effects of LPS are mediated through the release of cytokines such as TNFα (Tracey, 1991), IL-1 (Wakabayashi et al., 1991) and IFNγ (Bucklin

30   et al., 1994). Much of the evidence supporting the role of these cytokines as mediators of septic shock comes from lethality studies involving the blockade of individual cytokines, resulting in

protection of experimental animals from otherwise lethal doses of endotoxin or gram-negative bacteria. One of the first events in septic shock is the activation of T cells by antigen presenting cells onto which bacterial superantigen is bound (Miethke et al., 1993). Upon activation, for which co-stimulation of CD28 is essential (Saha et al., 1996), these T cells proliferate and produce a surge of proinflammatory cytokines such as IL-2, TNFα and IFNγ, eventuating in the clinical syndrome. Also, it is hypothesized that LPS induces the expression of the α1/β1 integrin (VLA-1) heterodimer on activated monocytes which then display an increased capacity to adhere to the endothelial basement membrane. Similar effects can be induced by incubation of monocytes with IFNγ (Rubio et al., 1995). VLA-1 might also contribute to further monocyte activation and potentiation of the production of monocyte-derived pro-inflammatory cytokines during sepsis (Rubio et al., 1995). Although very promising results were obtained with antibodies neutralising TNFα in experimental animal models, clinical trials with anti-TNFα antibodies revealed only a slight reduction or even no reduction in mortality rate of patients with septic shock (Wherry et al., 1993; Reinhart et al., 1996). A fusion protein containing the extracellular portion of the TNF receptor and the Fc portion of IgG1 also did not affect mortality (Fisher et al., 1996). Pentoxifylline (PTX), a methyl xanthine derivative, is currently being tested for its effect on the outcome of septic shock. PTX is known to lower the serum concentrations of at least TNFα, IL-1 and IFNγ (Bienvenu et al., 1995; Zeni et al., 1996). Initial data reveal that PTX leads to an improvement of the clinical status of septic patients (Mándi et al., 1995). There is evidence that IFNγ is a mediator of lethality during sepsis. Antibodies that either neutralize IFNγ or block the IFNγ-receptor are protecting against lethality (Bucklin et al., 1994; Doherty et al., 1992). A synergistic effect between IFNγ and TNFα has also been suggested (Doherty et al., 1992; Ozmen et al., 1994). Although not in itself lethal, IFNγ has been shown to be essential for the manifestation of TNF-induced lethality in the generalized Shwartzman reaction (Ozmen et al., 1994).

Bullous, inflammatory and neoplastic dermatoses are a heterogenous group of skin disorders during which IFNγ may play a pathogenic role. Bullous dermatoses encompass epidermolysis bullosa acquisita, bullous pemhigoid, dermatitis herpetiformes Duhring, linear IgA disease, herpes gestationis, cicatricial pemhigoid, bullous systemic lupus erythematosis, epidermolysis bullosa junctionalis, epidermolysis bullosa dystrophicans, porphyria cutanea tarda and Lyell-Syndrome (Megahed, 1996). Also erythema exsudativum multiform major (Kreutzer

et al., 1996), IgG-mediated subepidermal bullous dermatosis (Chan & Cooper, 1994), bullous lichen planus (Willsteed et al., 1991) and paraneoplastic bullous dermatosis (Pantaleeva, 1990) can be classified among the bullous dermatoses. A pathogenic role of IFNγ during bullous dermatoses has been suggested by Van den Oord et al. (1995). The role of IFNγ during

5   inflammatory and neoplastic dermatoses, compared to bullous dermatoses, has been more extensively investigated. Indeed, it has been demonstrated that IFNγ is involved during the pathogenesis of verrucosis (Asadullah et al., 1997), eosinophilic pustular folliculitis (Teraki et al., 1996), cutaneous T cell lymphoma (Wood et al., 1994), granuloma faciale (Smoller & Bortz, 1993), Sweet's syndrome (Reuss-Borst et al., 1993), atopic eczema (Arenberger et al., 1991) ,

10   follicular mucinosis (Meisnerr et al., 1991), lichen-planus and psoriasis (Vowels et al., 1994). One of the most extensively studied inflammatory dermatoses is psoriasis. Psoriasis is a hyperproliferative skin disorder affecting approximately 2% of the population. Evidence is accumulating that the disease has a T-cell mediated autoimmune etiology. The role of T-cells in psoriasis has been demonstrated by Gottlieb et al. (1995). The latter authors suggested that, in

15   most of the patients, clinical and histopathological features of psoriasis are primarily linked to skin infiltration by IL-2 receptor-positive leukocytes. Disease improvement can be induced by the administration of a fusion protein composed of human interleukin-2 and fragments of diphteria toxin, which selectively blocks the growth of activated lymphocytes. Other effective anti-psoriatic, T-cell suppressing agents include the immunosuppressive drugs cyclosporin and

20   FK506 (Griffiths, 1986) and anti-CD4 monoclonal antibodies (Morel et al., 1992). More direct evidence for the role of T cells in the induction of the complex tissue alterations seen in psoriasis has been generated by Schön et al. (1997) using a model with scid/scid mice in which they transferred naïve, minor histocompatibility mismatched CD4+ T-cells, resulting in the development of a skin disorder that resembles psoriasis. The autoimmune character of the disease

25   has been proposed by Valdimarsson et al. (1995) who stated that products of activated T-cells can induce keratinocytes of individuals with psoriatic predisposition to express determinants that are recognized by T cells specific for epitopes on β-haemolytic streptococci. Several data suggest that IFNγ may play a crucial role in the pathogenesis of psoriasis. IFNγ, produced by activated T cells would be involved in the recruitment of lymphocytes (Nickoloff, 1988), in the induction

30   of activation and adhesion molecules on epidermal keratinocytes (Dustin et al., 1988), as well as in the abnormal keratinocyte proliferation (Barker et al., 1993). Not only enhanced levels of

IFNγ has been detected in psoriatic epidermis (Kaneko et al., 1990), also de novo suprabasal expression of IFNγ receptor in psoriasis has been demonstrated (Van den Oord et al., 1995)

Inflammatory bowel disease (IBD), which encompasses ulcerative colitis and Crohn's disease, is characterized by the appearance of lesions of unknown aetiology in most parts of the gut. IBD is rather common, with a prevalence in the range of 70-170 in a population of 100,000. The current therapy of IBD involves the administration of anti-inflammatory or immunosuppressive agents, which usually bring only partial results, and surgery. In view of the apparent shortcomings of the present treatment, Ashkenazi and Ward (WO 94/14467) suggested the usage of a bispecific antibody construct targeting IFNγ and another molecule, such as IL-1 and TNFα, to treat IBD. However, the exact role of IFNγ during IBD is not well understood.

MS is a severely disabling progressive neurological disease of unknown aetiology, but probably involving autoimmune responses and resulting in the appearance of focal areas of demyelinisation (Williams et al., 1994). MS affects 1 in 1000 persons in the USA and Europe, but due to improved diagnosis that number is increasing. Onset of disease is usually around 30 years of age and, on average, patients are in need of treatment for another 28 years. MS is among the most expensive chronic diseases of western society, based on duration and intensity of care. However, diagnosis of exacerbations and early identification of onset of exacerbations has improved greatly, allowing design of novel treatment strategies. Active multiple sclerosis lesions feature T-lymphocyte and monocyte-macrophage accumulations at plaque margins where myelin is being destroyed. The inflammatory cells that invade the white matter and the soluble mediators that they release are held primarily responsible for myelin breakdown. Population-based studies indicate that certain HLA-antigens occur with higher frequency in patients with MS (with predominant MHC being the Dw2(DR2)DQ1.2 haplotype (Olerup et al., 1991). Similar associations of class I and class II haplotypes have also been detected in other autoimmune disorders such as rheumatoid arthritis and insulin dependent diabetes (Nepom, 1993). The lesions of MS are comparable to those found in chronic relapsing experimental allergic encephalitis (EAE), an autoimmune disease that can be induced in animals by immunization with e.g. whole myelin (Allen et al., 1993) or with the myelin/oligodendrocyte glycoprotein (Genain et al., 1995b). The lesions associated with EAE are similar in appearance as the ones occurring in MS and also contain inflammatory infiltrates of T-cells and macrophages (Genain et al., 1995b). Furthermore, in adoptive transfer experiments, T cells sensitized to specific myelin antigens can

transfer the disease state of EAE (Genain et al., 1995b; Waldburger et al., 1996). A few years ago, the American FDA approved the use of the immunosuppressive drug interferonβ (trade name Betaseron) for treatment of chronic relapsing MS. The effect of this drug - although modest - clearly demonstrates the involvement of the cytokine network in the pathophysiology of MS.

5   In the last few years, a large number of studies have addressed the molecular mechanism by which Betaseron exerts its beneficial effects. Lately, it was shown that IFNβ dose-dependently inhibited T-cell proliferation, expression of IL-2 receptors and secretion of IFNγ, TNFα and IL-13 (Rep et al., 1996). Furthermore, it was demonstrated that IFNβ could specifically prevent the IFNγ-induced up regulation of MHC class II antigens and adhesion molecules on antigen-

10   presenting cells (Jiang et al., 1995) and human brain microvessel endothelial cells (Huynh et al., 1995).

One of the earliest events in MS is damage of the blood brain barrier (BBB) by activated, encephalitogenic T-cells (Tsukada et al., 1993). The mechanism by which these cells destruct locally the BBB, which is mainly constituted of endothelial cells, is not elucidated, but it is

15   known that at the systemic level, local production of certain cytokines such as IFNγ enhance the capability of lymphocytes to adhere to endothelial cells (Yu et al., 1985; Tsukada et al., 1993). Also, on choroid plexus epithelial cells of EAE animals, an increased expression of ICAM-1 and VCAM-1 (Steffen et al., 1994), for which LFA-1 and VLA-4 are the natural ligands on lymphocytes, has been observed. Mc Carron et al. (1993) reported that adhesion of MBP-specific

20   T lymphocytes was significantly up regulated when cerebral endothelial cells were treated with IL-1, TNFα or IFNγ. That the adhesion of encephalitogenic T-cells to the endothelium is an early and very important event in the onset of MS is shown by the finding that anti LFA-1 therapy can completely block the induction of EAE (Gordon et al., 1995). Additional circumstantial evidence for a stimulatory role of IFNγ in the pathophysiology of MS comes from observations that

25   disease exacerbations are induced by viral upper respiratory infections, known to stimulate the secretion of IFNγ by type-2 helper T cells (Panitch, 1994). The proinflammatory role of IFNγ in autoimmune disease is strengthened by an earlier finding that treatment of MS patients with hIFNγ resulted in an aggravation of the symptoms (Panitch et al., 1986). The role of IFNγ as proinflammatory cytokine in autoimmune disorders has been studied in several experimentally

30   induced forms of autoimmunity. In experimental neuritis, induced by myelin or antigen-specific T cells in rat, IFNγ clearly acted as pro-inflammatory cytokine and administration of a

monoclonal antibody to IFNγ suppressed the disease (Hartung et al., 1990). In the case of experimental autoimmune thyroiditis (EAT) in mice, induced by the injection of thyroglobulin, treatment of the animals with anti-IFNγ at 4 weeks after induction of EAT proved to be beneficial, since characteristic features of EAT such as the lymphocytic infiltrations of the

5   thyroid glands and the serum levels of autoantibodies to thyroglobulin, were significantly reduced (Tang et al., 1993).

In the mouse EAE model for MS, where the disease can be induced by injection of either spinal cord homogenate or myelin basic protein, elevated concentrations of several cytokines, including IFNγ were observed both in serum and in the lesions in the CNS (Willenborg et al., 1995).

10   However, administration of anti-IFNγ at the initiation of the disease, resulted in an exacerbation of the disease (Billiau et al., 1988; Duong et al., 1994; Willenborg et al., 1995). It must be noted, however, that in these experiments the effect of anti-IFNγ was determined at the onset of acute EAE rather than at the time of chronic relapse of the disease, which in fact is the only relevant situation for MS. Pathologically, typical acute EAE differs substantially from MS in that

15   prominent inflammation occurs in gray, white and meningeal structures, but demyelisation is scant or absent (Genain et al., 1995b). In order to explain the findings with anti-IFNγ antibodies, the authors suggest a different action of IFNγ at the systemic level (anti-inflammatory action) compared to the local level (inflammatory action) (Billiau et al., 1988), or suggest an early role (within 24h after immunization) of IFNγ in disease resistance (Duong et al., 1994). Willenborg

20   et al. (1995) conclude that the time of treatment plays a critical role on the outcome and suggest this to be the explanation for conflicting results in different autoimmune processes. Recently, Heremans et al. (1996) described facilitation of spontaneous relapses in chronic relapsing EAE in Biozzi ABH mice by administration of anti-IFNγ during the remission phase. The onset of relapses was delayed when animals were treated with IFNγ during the remission phase, results

25   which are in contradiction to the excacerbation seen in humans who were treated with hIFNγ. An experimental EAE model that more closely resembles the disease course and symptomatology of MS in humans can be found in marmosets. Indeed, in these animals a chronic relapsing-remitting form of EAE can be induced which is characterized by an initial, acute phase with clinically mild neurological signs, followed by recovery. A late spontaneous relapse occurs

30   in these animals and chronic lesions resemble active plaques of chronic MS (Massacesi et al., 1995). This unique model can efficiently be employed to evaluate a prospective therapy for MS.

In this model, a critical role for TNFα in demyelisation is suggested by the observation that rolipram, a selective inhibitor of the type IV phosphodiesterase, suppressed TNFα secretion and demyelisation (Genain et al., 1995a; Sommer et al., 1995) when administered shortly after immunization, thus interfering with acute EAE. The effect of anti-IFNγ on acute EAE or on disease relapse has to our knowledge never been investigated in marmoset.

Taken together, it is well established that there are a number of clinical situations in which IFNγ-activity has deleterious effects. Consequently, several potential therapies to neutralize IFNγ-activity have been proposed. Among the latter proposals are the use of: anti-IFNγ antibodies (Ozmen et al., 1995; Bucklin et al., 1994), recombinant anti-IFNγ Fv fragments (EP 0528469 to Billiau & Froyen), bispecific molecules (WO 94/14467 to Ashkenazi and Ward), drugs such as pentoxifylline (Bienvenu et al., 1995), synthetic polypeptides which inhibit binding of IFNγ to its receptor (US 5,451,658 to Seelig; US 5,632,988 to Ingram et al.), Epstein-Barr virus derived proteins (US 5,627,155 to Moore & Kastelein), soluble IFNγ receptors (EP 0393502 to Fountoulakis et al.; US 5,578,707 to Novick & Rubinstein) and oligonucleotides which bind to IFNγ (WO95/00529 to Coppola et al.). However, these compounds are faced with problems such as suboptimal stability, affinity and clearance rates, lack of specificity, efficacy and tissue penetrance, toxic side effects and unwanted carrier effects. Indeed, the carrier effect of antibodies can limit their efficiency to block the target cytokine. For example, Montero-Julian et al. (1995) showed that during treatment of myeloma patients with anti-IL-6, accumulation of IL-6 in the serum in the form of monomeric immune complexes occurred, hereby stabilizing the cytokine. Furthermore, it has also been shown that the therapeutic efficacy of a cytokine can be prolonged by the formation of cytokine/antibody complexes, since the efficacy of recombinant human IL-2 treatment could be increased by prolonging its *in vivo* half-life by complexing with an anti-IL-2 antibody (Courtney et al., 1994). The carrier-effect of anti-cytokine antibodies can be overcome by the construction of monovalent scFv fragments, although their low MW (±30.000) and the associated fast clearance rate, make them less suitable candidates for long-term treatment. However, the undesirable carrier effect can be avoided by the formation of higher immune complexes, as such increasing the clearance of the cytokine-antibody complexes (Montero-Julian et al., 1995). The use of monoclonal antibodies for diagnostic or therapeutic purposes *in vivo* is, besides the carrier effect, also limited because of their nature (i.e. the majority are murine mAb's and administration of antibodies of mouse origin inevitably results

in a human anti-mouse antibody [HAMA] response), their suboptimal efficacy, stability and affinity and their large molecular size. Proposed solutions to some of these problems involve the use of F(ab')2, F(ab) and scFv derivatives or of humanized versions of the parent antibody, either by CDR grafting (Kettleborough et al., 1991) or by resurfacing of the antibodies (Roguska et al.,

5   1994). Another proposed solution is the development of several modified antibodies or antibody constructs by bioengineering or chemical methods. Indeed, some mAb's were made more effective by conjugating chemotherapeutic drugs and other toxins to the antibodies (Ghetie and Vitetta, 1994) or by developing bispecific and/or multivalent antibody constructs capable of simultaneously binding several -or two different epitopes on the same- or different antigens.

10   These antibody constructs have been produced using a variety of methods: a) antibodies of different specificities or univalent fragments of pepsin-treated antibodies of different specificities have been chemically linked (Fanger et al., 1992); b) two hybridomas secreting antibodies of different specificity have been fused and the resulting bispecific antibodies from the mixture of antibodies were subsequently isolated; c) genitically engineered single chain antibodies have

15   been used to produce non-covalently linked bispecific antibodies (e.g. diabodies (Holliger et al., 1993), minibodies (Kostelny et al., 1992) and tetravalent antibodies (Pack et al; 1995; WO 96/13583 to Pack) or covalently-linked bispecific antibodies (e.g. chelating recombinant antibodies (Kranz et al., 1995), single chain antibodies fused to protein A or Streptavidin (Ito and Kurosawa, 1993; Kipriyanov et al., 1996) and bispecific tetravalent antibodies (EP 0517024 to

20   Bosslet and Deeman). Recently, also trivalent antibody constructs, named triabodies (Kortt et al., 1997), and pentavalent constructs, named peptabodies (Terskikh et al., 1997), have been described. These constructs may have a higher avidity in comparison to bivalent constructs and may be useful for diagnostic or therapeutic purposes *in vivo*.

       However, and despite the fact that several potential therapies to neutralize IFNγ-activity

25   have been proposed, no prior art exists regarding the production and existence of engineered antibody constructs, such as humanized single-chain Fv fragments, diabodies, triabodies, tetravalent antibodies, peptabodies and hexabodies, and ruminant-derived antibodies such as sheep antibodies which overcome the above-indicated problems and which can efficiently be used to treat diseases wherein interferon-gamma activity is pathogenic.

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AIMS OF THE INVENTION

It is clear from the prior art as cited above that problems such as suboptimal stability, affinity, clearance rate, specificity, efficacy, and an unwanted carrier effect and HAMA response

5   hamper the successful usage of several therapeutics which, potentially, could neutralize the activity of IFNγ. Also suggested solutions to overcome some of these problems did not result in the development of effective products. Thus, unpredictable and unknown factors still appear to determine the success of these biologicals. Despite these unknown factors, the present inventors have been able to design and develop useful constructs which effectively neutralize

10   IFNγ-activity. Indeed, the constructs have all a surprisingly high affinity for IFNγ, they do not provoke a HAMA or related response, and they do not result in a carrier effect. In addition, some of the constructs pass the blood brain barrier, whereas others have a very good clearance rate. Therefore, the present invention aims at providing a molecule which binds and neutralizes interferon-gamma and which is chosen from the group consisting of:

15   - a scFv comprising the humanized variable domain of the monoclonal antibody D9D10
     - a chimeric antibody comprising the humanized variable domain of the monoclonal antibody D9D10
     - a diabody comprising the humanized variable domain of the monoclonal antibody D9D10
     - a multivalent antibody

20   - a ruminant antibody.

The present invention further aims at providing a multivalent antibody chosen from the group consisting of triabodies, tetravalent antibodies, peptabodies and hexabodies.

The present invention also aims at providing a triabody, tetravalent antibody, peptabody and hexabody which comprise 3, 4, 5 and 6 variable domains, respectively, of different anti-

25   interferon-gamma antibodies.

The present invention further aims at providing a triabody as described above which comprises 3 identical variable domains of an anti-interferon-gamma antibody. A preferred variable domain used in the latter constructs is derived from the mouse anti-interferon-gamma antibody D9D10 which is described by Sandvig et al. (1987) and Froyen et al. (1993) or from

30   the sheep anti-interferon-gamma antibody described in the present application. Therefore, the present invention aims at providing a triabody as described above which comprises 3 identical

D9D10 scFv's, 3 identical humanized D9D10 scFv's, 3 identical sheep-derived anti-interferon-gamma scFv's or 3 identical humanized sheep-derived anti-interferon-gamma scFv's.

The present invention further aims at providing a tetravalent antibody (called MoTAb I) as described above which comprises 4 identical domains of an anti-interferon-gamma antibody.

5   More specifically, the present invention aims at providing a tetravalent antibody as described above which comprises either 4 identical D9D10 scFv's or 4 identical sheep-derived anti-interferon-gamma scFv's in the format of a homodimer of 2 identical molecules, each containing 2 D9D10 scFv's or 2 humanized D9D10 scFv's or 2 sheep-derived anti-interferon-gamma scFv's or 2 humanized sheep-derived anti-interferon-gamma scFv's, and a dimerization domain, or, a

10   full-size humanized D9D10 antibody or sheep-derived anti-interferon-gamma antibody to which 2 humanized D9D10 scFv's or 2 humanized sheep-derived anti-interferon-gamma scFv's, respectively, are attached at the carboxyterminus (called MoTAb II) (see Fig. 1).

The present invention further aims at providing a peptabody and hexabody as described above which comprise 5 and 6 identical variable domains of an anti-interferon-gamma antibody,

15   respectively. A preferred variable domain used in the latter constructs is derived from the mouse anti-interferon-gamma antibody D9D10 which is described above or from the sheep anti-interferon-gamma antibody described in the present application. Therefore, the present invention aims at providing a peptabody and hexabody as described above which comprises 5 or 6 identical D9D10 scFv's, 5 or 6 identical humanized D9D10 scFv's, 5 or 6 identical sheep-derived anti-

20   interferon-gamma scFv's, or, 5 or 6 identical humanized sheep-derived anti-interferon-gamma scFv's, respectively.

The present invention further aims at providing a molecule as described above, wherein said ruminant antibody is a sheep antibody.

The present invention also aims at providing a molecule as described above, wherein said

25   sheep antibody is a monoclonal antibody. Furthermore, the present invention aims at providing a humanized antibody, a single-chain fragment or any other fragment which is derived from said monoclonal antibody and which has largely retained the specificity of said monoclonal antibody.

Moreover, the present invention aims at providing methods for producing the above-described molecules.

30   The present invention further aims at providing a pharmaceutical composition comprising a molecule as described above, or a mixture of said molecules, in a pharmaceutically acceptable

excipient.

The present invention also aims at providing a molecule or a composition as described above for use as a medicament.

Furthermore, the present invention aims at providing a molecule or a composition as
5   described above for preventing or treating septic shock, cachexia, immune diseases such as multiple sclerosis and Crohn's disease and skin disorders such as bullous, inflammatory and neoplastic dermatosis.

Finally, the present invention aims at providing a molecule as described above for determining interferon gamma levels in a sample.

10   All the aims of the present invention are considered to have been met by the embodiments as set out below.

BRIEF DESCRIPTION OF FIGURES

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Figure 1 schematically shows 2 different tetravalent antibody constructs (MoTAB I and MotabII). MoTAb I represents a molecule which consists of 4 identical scFv's in the format of a homodimer of 2 identical molecules, each containing 2 scFv's. MoTAb II represents a full-size antibody molecule to which 2 scFv's with the same specificity are attached at the
20   carboxyterminus. Optionally, these constructs contain a purification/detection tag.
See also further *Example 4*.

Figure 2 shows the coding and amino acid sequence of humanized D9D10 scFv (containing a C-terminal 6-histidine tag (bold)). CDR regions are underlined. Mutations (murine -> human)
25   are bold and underlined. The N-terminal pelB signal sequence is put in bold.

Figure 3 and 4 shows the binding of different concentrations of murine scFvD9D10 (figure 3) and humanized scFvD9D10 (figure 4) to human IFNγ. Human IFNγ is immobilized indirectly to the CM5 sensorchip via the murine D9D10 full size antibody as described in example 1.
30   Association rate constants derived from these binding curves are shown. Dissociation rate constants could not be measured accurately as dissociation is hardly detectable ($< 5 \times 10^{-4}$ s^{-1}) in

12

this experimental setup.

Figure 5 shows a schematic representation of the mammalian expression plasmid pEE12hD9D10
used for expression of humanized D9D10 whole antibody in (1) COS cells (2) stable recombinant

5   NsO cell lines.

Major plasmid building blocks :

* prokaryotic sequences for plasmid DNA preparation in E.coli (ori of replication and amp^R
ampicilline resistance expression unit)

* SV40 origin of replication (part of SV40E, SV40 early promoter) allowing transient expression

10   in SV40 permissive, T-antigen producing cell lines (e.g. COS)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron)
controlled expression casette for hD9D10 heavy chain protein (hD9D10-H)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron)
controlled expression casette for hD9D10 light chain protein (hD9D10-L)

15   * SV40 early promoter (SV40E) controlled glutamine synthetase cDNA (GS) expression unit
for selection/amplification

polyA= SV40 early region poly-adenylation signal

intron + polyA = SV40 t-antigen intron +SV40 early region poly-adenylation signal

20   **Figure 6** shows a schematic representation of the mammalian expression plasmid pEE14hD9D10
used for expression of humanized D9D10 whole antibody in (1) COS cells (2) stable recombinant
CHO-K1 cell lines.

Major plasmid building blocks :

* prokaryotic sequences for plasmid DNA preparation in E.coli (ori of replication and amp^R

25   ampicilline resistance expression unit)

* SV40 origin of replication (part of SV40E, SV40 early promoter) allowing transient expression
in SV40 permissive, T-antigen producing cell lines (e.g. COS)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron)
controlled expression casette for hD9D10 heavy chain protein (hD9D10-H)

30   * human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron)
controlled expression casette for hD9D10 light chain protein (hD9D10-L)

* SV40 late promoter (SV40L) controlled glutamine synthetase mini gene (GS+intron) expression unit for selection/amplification

polyA= SV40 early region poly-adenylation signal

intron + polyA = SV40 t-antigen intron +SV40 early region poly-adenylation signal

5

Figure 7 shows the cDNA sequence encoding the humanized D9D10 heavy chain fusion protein.

bp 1-60 : D9D10 Kappa-light chain signal sequence

bp 61-411 : humanized D9D10 heavy chain variable domain

bp 412-1401 : human IgG1 heavy chain constant domain (C_H1-Hinge-C_H2-C_H3)

10   bp 1402-1404 : leu codon added by PCR cloning strategy

Figure 8 shows the cDNA sequence encoding the humanized D9D10 and MoTAbII light chain fusion protein.

bp 1-60 : D9D10 Kappa-light chain signal sequence

15   bp 61-381: humanized D9D10 light chain variable domain

bp 382-699 : human kappa light chain constant domain

Figure 9 shows the amino acid sequence of the humanized D9D10 heavy chain fusion protein.

Aa 1-20 : D9D10 light chain signal sequence

20   Aa 21-137 : humanized heavy chain variable domain of D9D10

Aa138-467: human IgG1 heavy chain constant domain(C_H1-hinge-C_H2-C_H3)

Aa 468 : leu added by PCR cloning strategy

Aa 351 : pro was mutated to ser : inactivation C1q complement binding

Number of residues : 468.

25   Molecular weight (MW) : 51413

Figure 10 shows the amino acid sequence of the humanized D9D10 and MoTAbII light chain fusion protein.

Aa 1-20 : D9D10 light chain signal sequence

30   Aa 21-127 : humanized light chain variable domain of D9D10

Aa 128-233 : human kappa light chain constant domain

14

Number of residues : 233.

Molecular weight (MW) : 25582

Figure 11 shows the binding in ELISA of different concentrations of humanized D9D10 and humanized D9D10 MoTabII (= different dilutions of crude COS supernatant containing humanized D9D10 or humanized D9D10 MoTabII) to immobilized human IFNγ. The assay is performed as described in example 2.

Figure 12 shows the interaction of humanized D9D10 (= crude COS supernatant containing humanized D9D10) with IFNγ using SPR analysis. The assay is performed as described in example 2.

Figure 13 shows the binding in ELISA of different concentrations of purified humanized D9D10 and MoTabII to immobilized human IFNγ. The assay is performed as described in example 2.

Figure 14 shows a schematic representation of the expressionplasmid pMoTAblH6 used for the expression of MoTAbl in *E.coli*.

Figure 15 shows the cDNA sequence of MoTAbl

bp 1- 351   : V$_H$ D9D10

bp 352 - 396   : (G$_4$S)$_3$ linker

bp 397 - 717   : V$_L$ D9D10

bp 718 - 750   : human IgG3 upper hinge

bp 751 - 855   : helix-turn-helix dimerisation domain

bp 856 - 888   : human IgG3 upper hinge

bp 889 - 1239 : V$_H$ D9D10

bp 1240 - 1284: (G$_4$S)$_3$ linker

bp 1285 - 1605: V$_L$ D9D10

bp 1606 - 1623: His6 tag

Figure 16 shows the AA sequence of MoTAbI

aa 1- 117   : V$_H$ D9D10

aa 118 - 132   : (G$_4$S)$_3$ linker

aa 133 - 239   : V$_L$ D9D10

5   aa 240 - 250   : human IgG3 upper hinge

aa 251 - 285   : helix-turn-helix dimerisation domain

aa 286 - 296   : human IgG3 upper hinge

aa 297 - 413   : V$_H$ D9D10

aa 414 - 428   : (G$_4$S)$_3$ linker

10   aa 429 - 525   : V$_L$ D9D10

aa 526 - 531   : His6 tag

Figure 17 shows a schematic representation of the mammalian expression plasmid pEE12MoTAbII used for expression of D9D10MoTAbII recombinant antibody in (1) COS cells

15   (2) stable recombinant NsO cell lines.

Major plasmid building blocks :

* prokaryotic sequences for plasmid DNA preparation in E.coli (ori of replication and amp^R ampicilline resistance expression unit)

* SV40 origin of replication (part of SV40E, SV40 early promoter) allowing transient expression

20   in SV40 permissive, T-antigen producing cell lines (e.g. COS)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron) controlled expression casette for D9D10MoTAbII heavy chain protein (MoTAbII-H)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron) controlled expression casette for D9D10MoTAbII light chain protein (MoTAbII-L)

25   * SV40 early promoter (SV40E) controlled glutamine synthetase cDNA (GS) expression unit for selection/amplification

polyA= SV40 early region poly-adenylation signal

intron + polyA = SV40 t-antigen intron +SV40 early region poly-adenylation signal

30   Figure 18 shows a schematic representation of the mammalian expression plasmid pEE14MoTAbII used for expression of D9D10MoTAbII recombinant antibody in (1) COS cells

(2) stable recombinant CHO-K1 cell lines.

Major plasmid building blocks :

* prokaryotic sequences for plasmid DNA preparation in E.coli (ori of replication and ampR ampicilline resistance expression unit)

5   * SV40 origin of replication (part of SV40E, SV40 early promoter) allowing transient expression in SV40 permissive, T-antigen producing cell lines (e.g. COS)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron) controlled expression casette for D9D10MoTAbII heavy chain protein (MoTAbII-H)

* human Cytomegalovirus major immediate early promoter/enhancer (hCMVprom + intron)

10   controlled expression casette for D9D10MoTAbII light chain protein (MoTAbII-L)

* SV40 late promoter (SV40L) controlled glutamine synthetase mini gene (GS+ intron) expression unit for selection/amplification

polyA= SV40 early region poly-adenylation signal

intron + polyA = SV40 t-antigen intron +SV40 early region poly-adenylation signal

15

Figure 19 shows the cDNA sequence encoding the MoTABII fusion protein

bp 1-60 : D9D10 Kappa-light chain signal sequence

bp 61-411 : humanized D9D10 heavy chain variable domain

bp 412-1401 : human IgG1 heavy chain constant domain (C_H1-Hinge-C_H2-C_H3)

20   bp 1402-1404 : leu codon added by PCR cloning strategy

bp 1405-1416 : gly(3)-ser codon

bp 1417-2133 : humanized D9D10 ScFv

Figure 20 shows the amino acid sequence of MoTABII fusion protein

25   Aa 1-20 : mouse D9D10 light chain signal sequence

Aa 21-137 : humanized heavy chain variable domain of D9D10

Aa 138-467 : human IgG1 heavy chain constant domain(C_H1-hinge-C_H2-C_H3)

Aa 351 : pro mutated to ser : inactivation C1q complement binding

Aa 468 : leu added by cloning strategy

30   Aa 469-472 : gly(3)-ser linker

Aa 473-711 : humanized D9D10 ScFv (V_H473-490/gly-ser linker/V_L605-711)

Figure 21 shows the interaction of MoTAbII (= crude COS supernatant containing MoTAbII) with IFNγ using SPR analysis. The assay is performed as described in example 4.

Figure 22 shows the amino acid sequence of the D9D10 L10 diabody

5   aa 1- 117   : V_H D9D10

aa 118 - 127   : $(G_4S)_2$ linker

aa 128 - 234   : V_L D9D10

aa 235 - 240   : His6-tag

10   **Figure 23** shows the coding sequence of the D9D10 L10 diabody

bp 1- 351   : V_H D9D10

bp 352 - 381   : $(G_4S)_2$ linker

bp 382 - 702   : V_L D9D10

15   **Figure 24** shows the amino acid sequence of the D9D10 L5 diabody

aa 1- 117   : V_H D9D10

aa 118 - 122   : G_4S linker

aa 123 - 229   : V_L D9D10

aa 230 - 235   : His6-tag

20

Figure 25 shows the coding sequence of the D9D10 L5 diabody

bp 1- 351   : V_H D9D10

bp 352 - 366   : G_4S linker

bp 367 - 687   : V_L D9D10

25

Figure 26 shows the interaction of humanized L5 D9D10 diabody (= crude lysate from *E. Coli*) with IFNγ using SPR analysis. The assay is performed as described in example 5.

Figure 27 shows the coding sequence of the D9D10 L0 triabody

30   bp 1- 351   : V_H D9D10

bp 352 - 672   : V_L D9D10

Figure 28 shows the amino acid sequence of the D9D10 L0 triabody

aa 1- 117   : V_H D9D10

aa 118 - 224   : V_L D9D10

aa 225 - 230   : His6-tag

5

Figure 29 shows the interaction of humanized L0 D9D10 triabody (= crude lysate from *E. Coli*) with IFNγ using SPR analysis. The assay is performed as described in example 6.

Figure 30 shows the neutralization of IFN-gamma-induced MHC class II upregulation on human

10   primary keratinocytes by D9D10 or D9D10 scFv. Human keratinocytes were cultured for 24 h with or without (not shown) 100 U/ml huIFN-gamma in the absence or the presence of D9D10 (2μg/ml). Resting human keratinocytes do not express MHC class II. IFN-gamma induces expression of MHC class II in the keratinocytes and D9D10 (upper panel) or scFv D9D10 (lower panel) inhibit this IFN-gamma-induced MHC class II expression. See also further *Example 7.1.*

15

Figure 31 shows the neutralization of IFN-gamma-induced MHC class II upregulation on human primary keratinocytes by crude COS supernatant containing either humanized D9D10 or MoTAbII. The experiment was performed as described in figure 30

thin line : human keratinocytes treated with human IFNγ

20   bold line :   A : human keratinocytes not treated with human IFNγ

                      B : effect of 400 ng/ml murine D9D10

                      C : effect of humanized D9D10 (crude COS supernatant)

                      D : effect of MoTAbII (crude COS supernatant)

25   **Figure 32** shows the effect of the anti-IFN-gamma antibody F3 and scFvF3 on the survival of mice in which the lethal shock syndrome called "Shwartzman reaction" is induced. See also further *Example 7.3.*

Figure 33 shows the effect of the anti-IFN-gamma antibody F3 and scFvF3 on body weight of

30   mice exhibiting IFN-gamma induced cachexia. Mortality (number of dead mice/total number of mice) is shown between brackets and the symbol "+". See also further *Example 7.4.*

19

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending
5   patent applications. All of these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention is based on the finding that a molecule which binds and neutralizes human interferon-gamma and which is chosen from the group consisting of:

- a scFv comprising the humanized variable domain of the monoclonal antibody D9D10
10 - a chimeric antibody comprising the humanized variable domain of the monoclonal antibody D9D10

- a diabody comprising the humanized variable domain of the monoclonal antibody D9D10

- a multivalent antibody

- a ruminant antibody
15 is useful to treat diseases where IFNγ activity is pathogenic.

As used herein the terms "molecule which binds and neutralizes IFNγ" refer to a molecule which recognizes and binds any particular epitope of IFNγ resulting in the neutralization of any bioactivity of IFNγ. Particular epitopes of IFNγ relate to the so-called E2 epitope recognized and bound by the mAb D9D10 , the so-called E1 epitope (Kwok et al., 1993)
20 or any other epitope. IFNγ specifically relates to human IFNγ but may also relate to non-human primate, mouse, rat, sheep, goat, camel, cow, llama or any other IFNγ. Furthermore, the term "bioactivity of IFNγ" relates to the antiviral activity (Billiau, 1996), the induction of the expression of MHC-class-II molecules by macrophages and other cell types (Steinman et al., 1980), the stimulation of the production of inflammatory mediators such as TNFα, IL-1 and NO
25 (Lorsbach et al., 1993), the induction of the expression of adhesion molecules such as ICAM-1 (Dustin et al., 1988) and of important costimulators such as the B7 molecules on professional antigen presenting cells (Freedman et al., 1991), the induction of macrophages to become tumoricidal (Pace et al., 1983), the induction of Ig isotype switching (Snapper and Paul, 1987), any pathological and/or clinical activity during diseases where IFNγ is pathogenic (Billiau, 1996)
30 or any other known bioactivity of IFNγ. In this regard, it should be clear that any assay system demonstrating the IFNγ-neutralizing capacity of a molecule, such as the ones described by

20

Novelli et al. (1991), Lewis (1995) and Turano et al. (1992) can be used. Some of these assays
are also described in the subsection *Evaluation of anti-IFNγ neutralizing molecules* in the
Examples section of the present application (see further). It should be noted that the molecules
which bind and neutralize IFN-γ as described above neutralize at least one bioactivity, but not
5   necessarily all bioactivities, of IFN-γ.

        The present invention further relates to a scFv comprising the humanized variable domain
of the monoclonal antibody D9D10. As used herein, the term single-chain Fv, also termed single-
chain antibody, refers to engineered antibody constructs prepared by isolating the binding
domains (both heavy and light chain) of a binding antibody, and supplying a linking moiety
10   which permits preservation of the binding function. This forms, in essence, a radically
abbreviated antibody, having only the variable domain necessary for binding the antigen.
Determination and construction of single chain antibodies are described in US 4,946,778 to
Ladner et al. and in the *Examples* section of the present application (see further). The term
"humanized" means that at least a portion of the framework regions of an immunoglobulin or
15   engineered antibody construct is derived from human immunoglobulin sequences. It should be
clear that any method to humanize antibodies or antibody constructs, as for example by variable
domain resurfacing as described by Roguska et al. (1994) or CDR grafting or reshaping as
reviewed by Hurle and Gross (1994), can be used. The humanization of the scFv comprising the
variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section
20   of the present application. The monoclonal antibody D9D10 was prepared essentially as
described by Sandvig et al. (1987) and Froyen et al. (1993). It should also be noted that the
process of humanization of an antibody or antibody construct is regularly accompanied by a
significant loss in binding affinity of this antibody or antibody construct (Kettleborough et al.,
1991; Park et al., 1996 and Mateo et al., 1997). In contrast, and surprisingly, the constructs
25   humanized by the present inventors were not characterized by a significant loss in binding
affinity in comparison to their non-humanized counterparts.

        The present invention also relates to a chimeric antibody comprising the humanized
variable domain of the monoclonal antibody D9D10. The term "chimeric antibody" refers to an
engineered antibody construct comprising variable domains of one species (such as mouse, rat,
30   goat, sheep, cow, llama or camel variable domains), which may be humanized or not, and
constant domains of another species (such as non-human primate or human constant domains)

                                              21

(for review see Hurle and Gross (1994)). It should be clear that any method known in the art to develop chimeric antibodies or antibody constructs can be used. The generation of a chimeric antibody comprising the humanized variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application.

5   The present invention also concerns a diabody comprising the humanized variable domain of the monoclonal antibody D9D10. The term "diabody" relates to two non-covalently-linked scFv's, which then form a so-called diabody, as described in detail by Holliger et al. (1993) and reviewed by Poljak (1994). It should be clear that any method to generate diabodies, as for example described by Holliger et al. (1993), Poljak (1994) and Zhu et al. (1996), can be

10   used. The generation of diabodies comprising the variable domain of the monoclonal antibody D9D10 is described further in the *Examples* section of the present application.

It should also be clear that the scFv's, chimeric antibodies and diabodies described above are not limited to comprise the variable domain of the monoclonal antibody D9D10 but may also comprise variable domains of other anti-IFNγ antibodies, such as the sheep anti-IFNγ antibody

15   described further in the present application, which efficiently neutralize the bioactivity of IFNγ. Furthermore, the diabodies described above may also comprise two scFv's of different specificities. For example, the latter diabodies may simultaneously neutralize IFNγ on the one hand and may target another molecule, such as TNF-α, IL-1, IL-2, B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, tumour growth factor-beta (TGF-β), transferrin

20   receptor, insulin receptor and prostaglandin E2 or any other molecule, on the other hand.

The present invention also concerns multivalent antibodies which bind and neutralize IFNγ. As used herein, the term multivalent antibody refers to any IFNγ-binding and IFNγ-neutralizing molecule which has more than two IFNγ-binding regions. Examples of such multivalent antibodies are triabodies, tetravalent antibodies, peptabodies and hexabodies which

25   bind and neutralize IFNγ and which have three, four, five and six IFNγ-binding regions, respectively.

The present invention thus relates, as indicated above, to triabodies which bind and neutralize IFNγ. As used herein, the term "triabody" relates to trivalent constructs comprising 3 scFv's, and thus comprising 3 variable domains, as described by Kortt et al. (1997) and Iliades

30   et al. (1997). A method to generate triabodies is described by Kortt et al. (1997) and the generation of triabodies comprising the variable domain of the monoclonal antibody D9D10 is

described further in the *Examples* section of the present application. It should be noted that the triabodies of the present invention may comprise: 3 variable domains of 3 different anti-IFNγ Ab's (i.e. 3 anti-IFNγ Ab's which recognize and bind a different epitope on IFNγ [see also above]), 3 variable domains of 3 identical anti-IFNγ Ab's such as 3 variable domains of D9D10

5   or 3 variable domains of humanized D9D10 or 3 variable domains of sheep anti-IFNγ Ab's or 3 humanized variable domains of sheep anti-IFNγ Ab's, 1 or 2 variable domain(s) of anti-IFNγ Ab's in combination with 2 or 1 variable domain(s) of an Ab which binds to any other molecule than IFNγ, respectively. Examples of such other molecules comprise TNF-α, IL-1, IL-2, B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, tumour growth factor-beta

10   (TGF-β), transferrin receptor, insulin receptor and prostaglandin E2.

The present invention further relates to tetravalent antibodies which bind and neutralize IFNγ. As used herein, the term "tetravalent antibody" refers to engineered antibody constructs comprising 4 antigen-binding regions as described by Pack et al. (1995) and Coloma & Morrison (1997). Methods to generate these tetravalent antibody constructs are also described by the latter

15   authors. The generation of the following 2 different tetravalent antibodies comprising the variable domain of the monoclonal antibody D9D10 are described further in the *Examples* section of the present application: MoTabl which consists of 4 identical humanized D9D10 scFv's in the format of a homodimer of two identical molecules each containing two D9D10 scFv's which are linked together using a dimerization domain; the latter domain also drives the homodimerization

20   of the molecule, and, MoTab II which consists of a full-size humanized D9D10 molecule to which two humanized D9D10 scFv's are attached at the carboxyterminus (CH3-domain). It should be noted that the tetravalent antibodies of the present invention may comprise: 4 variable domains of 4 different anti-IFNγ Ab's (i.e. anti-IFNγ Ab's which recognize and bind to a different epitope on IFNγ), 4 variable domains of 4 identical anti-IFNγ Ab's such as 4 variable

25   domains of D9D10 or 4 variable domains of humanized D9D10 or 4 variable domains of sheep anti-IFNγ Ab's or 4 humanized variable domains of sheep anti-IFNγ Ab's, 2 variable domain(s) of one anti-IFNγ Ab in combination with 2 variable domain(s) of another anti-IFNγ Ab, 2 variable domain(s) of anti-IFNγ Ab's in combination with 2 variable domain(s) which binds to any other molecule than IFNγ. Examples of such other molecules comprise TNF-α, IL-1, IL-2,

30   B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, TGF-β, transferrin receptor, insulin receptor and prostaglandin E2.

Furthermore, the term "dimerization domain" of MoTab I refers to any molecule known in the art which is capable of coupling the two identical molecules. Examples of such domains are the leucine zipper domain (de Kruif & Logtenberg, 1996), the helix-turn-helix motif described by Pack et al. (1993), the max-interacting proteins and related molecules as described

5   in US 5,512,473 to Brent & Zervos and the polyglutamic acid-polylysine domains as described in US 5,582,996 to Curtis.

The present invention thus relates, as indicated above, to peptabodies and hexabodies which bind and neutralize IFNγ. As used herein, the term "peptabodies" relates to pentavalent constructs as described in detail by Terskikh et al. (1997). The term "hexabodies" relates to

10   hexavalent constructs which are similar to the pentavalent constructs as described in detail by Terskikh et al. (1997) but wherein the pentamerization domain is replaced by any hexamerization domain known in the art. A method to generate peptabodies is also described by Terskikh et al. (1997) and a method to generate hexabodies can be derived from the description by the latter authors. It should be noted that the peptabodies and hexabodies of the present invention may

15   comprise: 5 (relating to the peptabodies) or 6 (relating to the hexabodies) variable domains of 5 or 6 different anti-IFNγ Ab's (i.e. 5 or 6 anti-IFNγ Ab's which recognize and bind a different epitope on IFNγ [see also above]), 5 or 6 variable domains of identical anti-IFNγ Ab's such as 5 or 6 variable domains of D9D10, or, 5 or 6 variable domains of humanized D9D10, or, 5 or 6 variable domains of sheep anti-IFNγ Ab's, or, 5 or 6 humanized variable domains of sheep

20   anti-IFNγ Ab's, less than 5 or 6 variable domain(s) of any anti-IFNγ Ab's in combination with less than 5 or 6 variable domain(s) of an Ab which binds to any other molecule than IFNγ, respectively. Examples of such other molecules comprise TNF-α, IL-1, IL-2, B7.1 or CD80, B7.2 or CD86, IL-12, IL-4, IL-10, CD40, CD40L, IL-6, TGF-β, transferrin receptor, insulin receptor and prostaglandin E2.

25   The present in invention further relates to ruminant antibodies which bind and neutralize IFNγ. The term "ruminant" relates to animals belonging to the suborder Ruminantia of even-toed hoofed mammals (as sheep, goats, cows, giraffes, deer, llama, vicunas and camels) that chew the cud and have a complex 3- or 4-chambered stomach.

More specifically, the present invention relates to sheep antibodies which bind and

30   neutralize IFNγ. The term "sheep" relates to any of numerous ruminant mammals belonging to the genus *Ovis*. The generation of sheep anti-IFNγ antibodies is described in the *Examples*

section of the present application. The present invention also relates to sheep monoclonal antibodies. As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. Indeed,

5   the monoclonal sheep antibodies of the present invention can be generated by any method known in the art. It should be noted that also humanized antibodies, scFv's or any other fragment thereof which has largely retained the specificity of said sheep antibody or sheep monoclonal antibody are covered by the present invention. As used herein, the term "fragment" refers to F(ab), F(ab')2, Fv, and other fragments which retain the antigen binding function and specificity of the

10   parent antibody. It should also be understood that the variable domains of the sheep anti-IFNγ (monoclonal) antibodies or scFv of the sheep anti-IFNγ (monoclonal) antibodies may be part of the chimeric antibodies, diabodies, triabodies, tetravalent antibodies, peptabodies and hexabodies as described above.

            The present invention further relates to scFv's, chimeric antibodies, diabodies, triabodies,

15   tetravalent antibodies, peptabodies, hexabodies and sheep antibodies which bind and neutralize IFNγ and which are produced by the methods as described above and in the *Examples* section of the present application.

            The present invention further relates to a composition comprising scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies

20   and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ in a pharmaceutically acceptable excipient, possibly in combination with other drugs or other antibodies, antibody derivatives or constructs for use as a medicament to prevent or treat septic shock, cachexia, immune diseases such as multiple sclerosis and Crohn's disease and skin disorders such as bullous, inflammatory and neoplastic dermatoses. Examples of such other drugs or other

25   antibodies, antibody derivatives or constructs are, with regard to septic shock: an isotonic crystalloid solution such as saline, dopamine, adrenaline and antibiotics; with regard to cachexia: anti-TNF-alpha antibodies; with regard to multiple sclerosis: ACTH and corticosteroids, interferon beta-1b (Betaseron), interferon beta-1a (Avonex), immunosuppressive drugs such as azathioprine, methotrexate, cyclophosphamide, cyclosporin A and cladribine (2-CdA),

30   copolymer 1 (composed of 4 amino acids common to myelin basic proteins), myelin antigens, roquinimex A, the mAb CAMPATH-1H and potassium channel blockers; with regard to Crohn's

disease: sulfasalazine, corticosteroids, 6 mercaptopurine/azathioprine and cyclosporin A; with regard to psoriasis: cyclosporin A, methotrexate, calcipotriene (Dovonex), zidovudine (Retrovir), histamine2 receptor antagonists such as ranitidine (Zantac) and cimetidine (Tagamet), propylthiouracil, acitretin (Soriatane), fumaric acid, vitamin D derivates, tazarotene (Tazorac),

5   IL-2 fusion toxin, tacrolimus (Prograf), CTLA4Ig, anti-CD4 mAb's and T-cell receptor peptide vaccines. It should also be clear that any possible mixture of the above-indicated IFN-γ-binding molecules may be part of the above-indicated pharmaceutical composition.

As used herein, the term "composition" refers to any composition comprising as an active ingredient scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent

10   antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ according to the present invention possibly in the presence of suitable excipients known to the skilled man. The scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ of the invention may thus be administered in the form

15   of any suitable composition as detailed below by any suitable method of administration within the knowledge of a skilled man. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable excipient. Such excipients are inherently nontoxic and

20   nontherapeutic. Examples of such excipients are saline, Ringer's solution, dextrose solution and Hank's solution. Nonaqueous excipients such as fixed oils and ethyl oleate may also be used. A preferred excipient is 5% dextrose in saline. The excipient may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

25   The scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ of the invention are administered at a concentration that is therapeutically effective to treat or prevent septic shock, cachexia, immune diseases such as multiple sclerosis and Crohn's disease and skin disorders such as bullous, inflammatory and neoplastic dermatoses.

30   The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that the scFv's and/or chimeric antibodies and/or diabodies

26

and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ are given at a dose between 1 μg/kg and 10 mg/kg, more preferably between 10 μg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg for each IFN-γ-binding molecule. Preferably, they are given as a bolus dose. Continuous short time

5   infusion (during 30 minutes) may also be used. If so, the scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ or compositions comprising the same may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute (for each IFN-γ-binding molecule).

10   According to the specific case, the "therapeutically effective amount" of a scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies and/or sheep antibodies which bind and neutralize IFNγ needed should be determined as being the amount sufficient to cure the patient in need of treatment or at least to partially arrest the disease and its complications. Amounts effective for such use will

15   depend on the severity of the disease and the general state of the patient's health. Single or multiple administrations may be required depending on the dosage and frequency as required and tolerated by the patient.

The present invention further relates to scFv's and/or chimeric antibodies and/or diabodies and/or triabodies and/or tetravalent antibodies and/or peptabodies and/or hexabodies

20   and/or sheep antibodies which bind and neutralize IFNγ for determining IFNγ levels in a biological sample, comprising:

1) contacting the biological sample to be analysed for the presence of IFNγ with a scFv and/or chimeric antibody and/or diabody and/or triabody and/or tetravalent antibody and/or peptabodies and/or hexabodies and/or sheep antibody as defined above,

25   2) detecting the immunological complex formed between IFNγ and said scFv and/or chimeric antibody and/or diabody and/or triabody and/or tetravalent antibody and/or peptabodies and/or hexabodies and/or sheep antibody.

As used herein, the term "a method to detect" refers to any immunoassay known in the art such as assays which utilize biotin and avidin or streptavidin, ELISA's and

30   immunoprecipitation, immunohistochemical techniques and agglutination assays. A detailed description of these assays is given in WO 96/13590 to Maertens & Stuyver. The

27

immunohistochemical detection of IFNγ in cryosections of spinal cord and brain of non-human

primates suffering from experimental autoimmune encephalomyelitis is described in detail in the

Examples section of the present application. The term "biological sample" relates to any possible

sample taken from a mammal including humans, such as blood (which also encompasses serum

5   and plasma samples), sputum, cerebrospinal fluid, urine, lymph or any possible histological

section, wherein IFNγ might be present.

The present invention will now be illustrated by reference to the following examples

which set forth particularly advantageous embodiments. However, it should be noted that these

embodiments are illustrative and are not to be construed as restricting the invention in any way.

10

EXAMPLES

1. Generation of humanized scFvD9D10

15

As the use of mouse monoclonals in humans induces a HAMA response, a humanized

antibody or antibody derivative is the alternative. Humanized scFvD9D10 need to have similar

binding and neutralization properties as their original mouse counterparts, but will elicit hardly

any immune response in humans as compared to the parent mouse scFv.

20

1.1. Modelling

We used computer modelling techniques for the construction of a humanized scFvD9D10

in order to develop an active scFv with retained structure and affinity. The scFv was humanized

25   using a resurfacing strategy which includes the replacement of 'non-human' residues without

significant structural changes of the scFv molecule. This work consisted of 2 main parts. In the

first part, a 3D-structure of the mouse scFv was constructed. For this purpose, we have

homology-modeled D9D10 using Ig V_L and V_H domains with a similar sequence and a known

structure. In the second part (the actual humanization step), we have aligned D9D10 with similar

30   *human* sequences to identify 'typically human residues'. After verifying their structural

compatibility with the D9D10 model, they have been proposed as residues-to-be-humanized.

PART 1 : 3D-structure of scFvD9D10

Identification of known structures with the most resembling sequence

5   DifferentBLAST-searches(http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-blast)were

performed by entering the D9D10 sequence of either V_K or V_H, by using the 'BLASTP' search

program and by selecting the Brookhaven Protein Data Bank (http://www.pdb.bnl.qov) as the

database to be searched. This search was performed 4 times, namely for V_K with and without

CDR-loops and for V_H with and without CDR-loops. The obtained data are summarized in

10   Table1.

Table 1. *Summary of BLAST-search results*

A) *BLAST-search using D9D10-V_K sequence*

rank	PDB code	score + CDR ident./sim.	score - CDR ident./sim.	rank for V_H	source	I.D.
1	1BAF	87%/92%	90%/95%	>50	mouse	Fab frag. mAb AnO2 compl. w. its hapten (2,2,6,6-Tetramethyl-1-Piperidinyloxy-Dinitrophenyl)
2	1FOR	80%/90%	85%/93%	16	mouse	Igg2a Fab frag. (Fab17-Ia)
3	2IFF	78%/86%	84%/90%	15	mouse	Igg1 Fab Frag. (Hyhel-5) compl. w. Chicken Lysozyme mutant R68K
4	1FIG	75%/86%	80%/90%	28	mouse	Chain L, Immunogl G1 (Kappa Light Chain) Fab' frag, Mouse
5	1FVB	80%/87%	83%/89%	>50	mouse	IgA Fv frag. (Anti-Alpha(1->6) Dextran) (Theoret. Model)
6	2HFL	77%/85%	83%/89%	14	mouse	IgG1 Fab frag. (HyHEL-5) compl. w. Chicken Lysozyme
...	...	...	...	...	...	...
19	1NCA	60%/73%	70%/84%	1	mouse	N9 neuraminidase-NC41 compl. w. Influenza Virus
...	...	...	...	...	...	...

B) *BLAST-search using D9D10-V$_H$ sequence*

rank	PDB code	score + CDR ident./sim.	score - CDR ident./sim.	rank for V$_K$	source	I.D.
1	1NCA	83%/89%	91%/95%	19	mouse?	N9 neuraminidase-NC41 compl. w. Influenza Virus
2	1NCB	80%/88%	87%/94%	>50	mouse?	N9 Neuraminidase-Nc41 Mut. N329D compl. w. Fab, Influenza Virus
3	1TET	80%/86%	87%/92%	38	mouse	Iggl Monocl. Fab frag (Te33) compl. w. Cholera Toxin Peptide 3
4	1DBA	80%/87%	86%/92%	>50	mouse	Fab' frag. of the Db3 Anti-Steroid Monocl. Ab
...	...	...	...	...	...	...
16	1FOR	58%/76%	63%/83%	2	mouse	Igg2a Fab frag. (Fab17-Ia)
...	...	...	...	...	...	...

A sequence similarity of more than 70% guarantees a strong structural similarity. For V$_K$, at least 6 very good matching structures (all murine proteins) could be identified: 1BAF, 1FOR, 2IFF, 1FIG, 1FVB and 2HFL. The scores for the search with CDR-loops varied from 87% to 77% for identical residues, and from 92% to 85% for chemically similar residues. The scores for the search without CDR-loops ranged from 90% to 83% identical residues and from 95% to 89% similar residues. The small difference in homology between the searches with and without CDR-loops suggests that even some of the CDR-loops are structurally similar. For V$_H$, analogous results were obtained. Four very well matching structures could be identified: 1NCA, 1NCB, 1TET and 1DBA with scores varying from 83% to 80% identical residues and from 89% to 87% similar residues when CDR-loops are included. If CDR-loops were not taken into account, significantly higher scores were obtained: from 91% to 86% for identical residues and 95% to 92% for similar residues. The latter was due to the fact that CDR-H3 from D9D10 was not matching well with any sequence.

Three-dimensional fitting of the best candidates

From these scores, it was clear that the V$_K$-fragment from 1FOR resembled very well V$_K$ from D9D10 (rank nr 2). A reasonably well homology was also found for its V$_H$ counterpart (rank nr 16). For the heavy domain, 1NCA had a very high score for V$_H$ (rank nr 1) and an

30

acceptable score for its V_K-domain (rank nr 19). Since the β-barrels of Fv fragments are well conserved, and since for both V_K and V_H we dispose of two very good resembling fragments with fairly well matching counterparts, we had enough information to start the construction of the D9D10 model.

5   When superimposing (fitting) the complete main chain of 1FOR and 1NCA we obtained a root-mean-square (rms) deviation of 1.1 Å (values around or less than 1 Å indicate a strong structural similarity). Fitting on V_K alone gave 1.0 Å and on V_H we obtained 0.8 Å. This means that both the complete structures and the separate V-domains are nearly identical. In order to obtain an even smaller rms-deviation, we fitted all β-strands of the central β-barrel, giving an

10   rms-deviation of 0.52 Å. When the C-terminal strands and certain diverging residues were not taken into account, an rms-deviation as low as 0.37 Å was obtained. The high structural resemblance of the central β-barrel of both 1FOR and 1NCA ensures us that we have correctly positioned the two domains relative to each other.

       In the next step, only the V_K fragment of 1FOR and the V_H of 1NCA were retained and

15   CDR-loops of 1FOR and 1NCA were adopted without further modeling.

Modeling of the D9D10 sequence onto the constructed framework

       When the sequences of D9D10 were compared with those of 1FOR-V_K and 1NCA-V_H, 21 and 20 mutations were necessary to mutate 1FOR and 1NCA into D9D10, respectively. These

20   mutations were done simultaneously using the Dead-End Elimination method (Desmet et al., 1992) which found the globally best conformation for all 41 mutations. For both V_K and V_H, the mutations could be done without inducing sterical or energetical conflicts. As a consequence, we have obtained a very reliable 3D-model for the variable domains of D9D10 (except for CDR-H3).

25

       * *PART 2 : Humanization of D9D10*

Identification of residues to be humanized

       In order to identify typical D9D10 'murine' residues, V_K and V_H sequences were again

30   subjected to a BLASTP-search, but this time the entire 'non-redundant Genbank' database (PDB + SwissProt + SPupdate + PIR) was searched for similar sequences. Out of the resulting matches,

only human and humanized sequences were retained and aligned with D9D10.

The alignment revealed several systematic differences in sequence between the murine D9D10 molecule and the best matching human V_K and V_H fragments. From this comparison, we have derived a consensus list of human residues.

5   Each of these residues was then placed onto the D9D10 model and the following properties were examined: (i) the compatibility with the framework and with neighboring residues, (ii) the solvent accessibility and (iii) the proximity to the CDR-loops. In general, only D9D10 residues which were not found in any human sequence, which were structurally compatible with the D9D10 framework (and CDR's), and which were clearly solvent exposed,

10   were selected for humanization.

For the V_K domain we proposed 8 mutations, which were spatially clustered into 2 surface patches of 3 residues each plus two isolated residues. For the V_H domain we pinpointed 9 residues to be humanized. The latter residues formed a surface cluster of 5 residues, one of 2 residues and 2 additional isolated residues. For neither of the two domains, buried residues were

15   retained in the mutation list. The reason for this is that we explicitly wanted to preserve the D9D10 framework structure and, also, that buried residues are not 'visible' to the immune system anyway.

Finally, the side-chain conformation of the 8+9 mutations was modeled using the Dead-End Elimination algorithm. We found that all mutations were energetically favorable. This

20   strengthened the hypothesis that the humanization procedure would not affect the antigen binding properties of D9D10.

1.2. Construction, expression, purification and evaluation of humanized scFvD9D10

25   Eight substitutions in V_HD9D10 and 9 in V_LD9D10 had to be carried out as shown in figure 2. Since the different mutations were spread among the whole V_H and V_L sequences, it was decided to assemble the whole V_H and V_L sequences out of synthetic oligonucleotides, hereby including all necessary substitutions during the oligonucleotide synthesis as an alternative to mutagenesis.   During the oligonucleotide synthesis, non-optimal *E.coli* codons were

30   substituted for more optimal ones coding for the same amino acid. Both V_H and V_L regions were assembled separately according to the PCR assembly method described by Stemmer *et al.*

(1995). The assembled V$_H$ and V$_L$ regions were first subcloned in pGEM-T vectors (PROMEGA Corp., Madison WI, US) and their correct sequence was confirmed by DNA sequencing. Both humanised regions were subsequently introduced into the pscFvD9D10H6 expression vector (Froyen et al., 1993). For the assembly of the heavy chain, we synthesized 18 oligo's, 40 nucleotides in length, which collectively encode both strands of the V$_H$ region from the AlwNI site to the StyI site. The plus strand as well as the minus strand consist of 9 oligo's configured in such a way that, upon assembly, complimentary oligo's will overlap by 20 nucleotides. In these oligo's we included mutations both leading to "humanised" amino acids at the predetermined sites and to "optimised" *E. coli* codons.

Oligo No.	Oligo Seq.
1s	5'-CGCGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAG-3'
2as	5'-CAGCTGCACCTGGGCCATCGCTGGTTGGGCAGCGAGTAAT-3'
3s	5'-CGATGGCCCAGGTGCAGCTGGTGCAGAGCGGTAGCGAACT-3'
4as	5'-CGCTCGCACCCGGTTTTTTCAGTTCGCTACCGCTCTGCAC-3'
5s	5'-GAAAAAACCGGGTGCGAGCGTTAAGATCAGCTGCAAAGCG-3'
6as	5'-TCGGTGAAGGTATAACCGCTCGCTTTGCAGCTGATCTTAA-3'
7s	5'-AGCGGTTATACCTTCACCGATTACGGTATGAACTGGGTTA-3'
8as	5'-ACCTTGACCCGGCGCCTGTTTAACCCAGTTCATACCGTAA-3'
9s	5'-AACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGGAT-3'
10as	5'-TTTCACCGGTGTAGGTGTTGATCCAACCCATCCATTTCAG-3'
11s	5'-CAACACCTACACCGGTGAAAGCACCTACGTTGACGATTTC-3'
12as	5'-CTGAAAACGAAACGACCTTTGAAATCGTCAACGTAGGTGC-3'
13s	5'-AAAGGTCGTTTCGTTTTCAGCCTGGATACCAGCGTTAGCG-3'
14as	5'-GCTGATCTGCAGGTAGGCCGCGCTAACGCTGGTATCCAGG-3'
15s	5'-CGGCCTACCTGCAGATCAGCTCTCTGAAAGCGGAAGACAC-3'
16as	5'-GCGCGCAGAAGTAGGTCGCGGTGTCTTCCGCTTTCAGAGA-3'
17s	5'-CGCGACCTACTTCTGCGCGCGTCGCGGTTTCTACGCGATG-3'
18as	5'-GCGCCCTTGGCCCCAGTAATCCATCGCGTAGAAACCGCGAC-3'

After assembly of the 18 40-mer oligonucleotides, the desired fragment was PCR amplified using 2 oligonucleotides complementary to the 5' and 3' end of the fragment respectively.

Oligo No.	Oligo Seq.
1s	5'-CGCGCAGCCGCTGGATTGTTATTAC-3'
2as	5'-GCGCCCTTGGCCCCAGTAATC-3'

The resulting 381 bp fragment was cloned into a pGEM-T vector, resulting in pGEM-TV$_H$H and several clones were sequenced. A similar approach was followed for the light chain. Hereby 14 oligos were synthesized, 2 48-mers and 12 40-mers, which collectively encode both strands of the V$_L$ region from the SacI site to the XhoI site. However, since the SacI site was present exactly on an amino acid substitution site, this restriction site could not be retained in the synthetic V$_L$ gene. As an alternative, a Bst1107I site was created which will, after ligation with the blunted SacI site, restore the exact V$_L$ reading frame.

Oligo No.	Oligo Seq.
1s	5'-CGCGGTATACTGACCCAGAGCCCGGCGACCATGAGCGCGAGCCCGGGT-3'
2as	5'-CAGGTCAGGGTAACACGTTCACCCGGGCTCGCGCTCATGG-3'
3s	5'-GAACGTGTTACCCTGACCTGCAGCGCGAGCTCTAGCATCA-3'
4as	5'-ATGATACCAGAACATATAGCTGATGCTAGAGCTCGCGCTG-3'
5s	5'-GCTATATGTTCTGGTATCATCAGCGTCCGGGTCAGAGCCC-3'
6as	5'-TATCATAGATCAACAGACGCGGGCTCTGACCCGGACGCTG-3'
7s	5'-GCGTCTGTTGATCTATGATACCAGCAACCTGGCGAGCGGT-3'
8as	5'-CCGCTGAAACGCGCCGGAACACCGCTCGCCAGGTTGCTGG-3'
9s	5'-GTTCCGGCGCGTTTCAGCGGTAGCGGTAGCGGTACCAGCT-3'
10as	5'-ACGGCTGATGGTCAGGCTATAGCTGGTACCGCTACCGCTA-3'
11s	5'-ATAGCCTGACCATCAGCCGTATGGAACCGGAAGATTTCGC-3'
12as	5'-TCTGATGGCAGAAATAGGTCGCGAAATCTTCCGGTTCCAT-3'

13s   5'-GACCTATTTCTGCCATCAGAGCTCTAGCTATCCGTTCACC-3'

14as   5'-CGCGCTCGAGTTTGGTACCCTGACCGAAGGTGAACGGATAGCTAGAGC-3'

After assembly of the 2 48-mer and 12 40-mer oligonucleotides, the desired fragment was

5   again PCR amplified using 2 oligonucleotides complementary to the 5' and 3' end of the fragment respectively.

Oligo No.	*Oligo Seq.*
1s	5'-CGCGGTATACTGACCCAGAGC-3'
2as	5'-CGCGCTCGAGTTTGGTACCCTG-3'

The resulting 316 bp fragment was cloned into a pGEM-T vector, resulting in pGEM-TV$_L$H and several clones were sequenced. The assembly PCR protocol (Stemmer et al., 1995) consisted of 3 steps: gene assembly, gene amplification and cloning. Since single-stranded ends of complementary DNA fragments were filled-in during the gene assembly process, cycling with *Taq* DNA polymerase resulted in the formation of increasingly larger DNA fragments until the full-length gene was obtained. It can be noted that DNA ligase has not been used in the process. After assembly, the desired fragments were amplified using 5' and 3' end complementary primers. The resulting fragments were subsequently cloned into a suitable cloning vector such as pGEM-T, giving pGEM-TV$_L$H and pGEM-TV$_H$H. The final vector, pscFvD9D10V$_{Hum}$, was constructed by ligating a 310 bp Bst1107I/XhoI fragment originating from vector pGEM-TV$_L$H with a 3180 bp SacIblunt/XhoI fragment originating from vector pscFvD9D10H6V$_H$H (= pscFvD9D10H6 in which V$_H$ was replaced by the humanized V$_H$ obtained from pGEM-TV$_H$H).

Induction of the humanised scFv D9D10 was carried out in *E.coli* strain JM83. Detection of His6-tagged scFv's on western blot was done with an anti D9D10 rabbit polyclonal antibody and an anti His6 monoclonal antibody (Babco, Richmond, CA, USA). Compared to the non-humanized scFvD9D10 (Froyen et al., 1993), the humanized scFvD9D10 was expressed at approximately 3-5 times higher levels (30-40 mg/l). This increase in expression level can be due to the fact that during assembly the humanized scFvD9D10 coding sequence was codon-optimised for *E. coli* expression. Alternatively, one or several of the humanized amino acids can have a beneficial effect on the expression level; or the increase in expression level can be caused

by a combination of the two. As with the non-humanized scFv, most of the expressed protein was still present intracellularly (70-80%), with 5-10% present in the periplasmic fraction and 10-20% secreted to the medium.

The cells were harvested and lysed in the presence of protease inhibitors at 4°C by the French press (2 passages at 14.000 psi). The cell lysate was clarified by centrifugation and the supernatant was used for purification. The supernatant was loaded on Zn^{2+}-IDA Sepharose FF and the resin was washed by applying an imidazole step gradient. The different pools were analysed by SDS-PAGE under reducing and non reducing conditions.

The humanized scFv bound and eluted as expected in the 150 mM imidazole elution pool and SDS-PAGE showed that the recovered scFv was >90% pure in a single step. The shift in relative migration under reducing conditions showed that the scFv was purified in an oxidized form. However, in contrast to the mouse scFv, the humanized scFv showed a high tendency for non specific adsorption, because only 40-50% of the initial product was recovered after dialysis.

The humanized scFvD9D10 was shown to have the same biological activity as the mouse scFvD9D10 for neutralizing the antiviral activity of human IFNγ (described in example 7).

Affinity could be calculated for murine and humanized scFv using Surface Plasmon Resonance(SPR)-analysis with the BIACORE® (Biacore AB, Uppsala, Sweden). This technology permits real-time mass measurements using surface plasmon resonance. SPR is an optical phenomenon, seen as a sharp dip in the intensity of light reflected from a thin metal film coated onto a glass support. The position of this dip depends on the concentration of solutes close to the metal surface. In general, a protein (e.g. antibody) is coupled to the dextran layer (covering the gold film) of a sensor chip and solutions containing different concentrations of a binding protein (e.g. antigen) are allowed to flow across the chip. Binding (association and dissociation) is monitored with mass sensitive detection.

In order to determine the affinity of the D9D10 derivatives for hIFNγ, BIACORE® experiments were performed in which the murine D9D10 was immobilized onto a CM5 sensorchip (Biacore AB). D9D10 was immobilized using amine coupling according to the manufacturer's procedure. To decrease the non specific interaction of human IFNγ with the carboxylic groups of the dextran layer, the sensorchip was pretreated with 4 cycles of EDC/NHS - thus reducing the amount of unblocked carboxylic groups remaining on the sensor surface - before immobilizing D9D10. Then, immobilization of D9D10 was carried out using a

continuous flow of 5μl/min on a sensor chip surface initially activated with 17 μl of an 0.05M

NHS/ 0.2M EDC mixture. 35μL of typically 3μg/ml D9D10 was injected over the activated

surface. Residual unreacted ester groups were blocked by injecting 17 μl of 0.1M ethanolamine

pH 8.5. D9D10 was immobilised directly on a CM5 chip at an optimal concentration of 3 μg/ml

5   in an acetate buffer pH 5.4 resulting in an immobilization level of about 600 RU. Most accurate

affinity data were obtained by injecting human IFNγ and monitoring the subsequent binding of

scFvD9D10; the latter interacting with remaining free epitopes on human IFNγ. On and off rates

were calculated using the BIAevaluation software (Biacore AB).

      Results of a typical experiment are shown in figure 3 for murine scFvD9D10 and in

10   figure 4 for humanized scFvD9D10 (These data were generated in separate experiments).

Calculated data were in good agreement. As off rates were hardly detectable for both constructs

in most experiments, only on rates are shown for the concentrations tested. These data clearly

indicated that the humanization did not hamper the binding characteristics of the scFv fragment.

      Monoclonal antibodies were generated against the humanized scFvD9D10. A female

15   BALB/c mouse was immunized (injected intraperitoneallly) 3 times with humanized scFvD9D10

(*i.e., at days 0 (50 μg), 32(25 μg) and 56(25 μg)*). Three months after, a final boost of 25 μg was

given. Three days after this last injection, spleen cells were retrieved from the immunized mouse

and used for cell fusion. Dissociated splenocytes from the immunized mouse were fused with

murine myeloma cells SP2/0-Ag14 (ATCC, CRL-1581) at a ratio of 10:3 using a polyethylene

20   glycol/DMSO solution mainly according the procedure as described by Köhler and Milstein

(1975). The fused cells were mixed up and resuspended in DMEM medium supplemented with

hypoxanthine, sodium pyruvate, glutamine, a non-essential amino acid solution, 20% heat-

inactivated fetalclone (Hyclone Lab., Utah) and 10% BM-Condimed (Boehringer Mannheim).

The cells were then distributed to 96 well plates to which aminopterin was added 24 hours after

25   the cell fusion. Each well contained between 1 to 5 growing hybridoma clones at the average.

After 8 days supernatant of the wells was collected and screened in an ELISA for binding to

humanized scFvD9D10. The antibodies of the hybridomas thus generated were further tested for

their binding capacity to murine and humanized scFvD9D10 and human IgG. Certain

monoclonal antibodies derived from this hyper immune mouse did recognize not only humanized

30   scFvD9D10 but also human IgG, indicating the quality of the humanization strategy. Using the

antibodies which specifically interact with humanized scFvD9D10 (1D5C5; 11E2G6; 10F12A2

available at Innogenetics N.V., Industriepark Zwijnaarde 7, Box 4, B-9052 Ghent, Belgium) and do not cross react with the yet tested human IgG preparations, an ELISA is generated for detecting and quantifying D9D10 derived constructs in human and primate serum.

5　　　　Immunization experiments in rabbit and mouse with his-tagged proteins including the humanized scFvD9D10 revealed weak to fairly high immunogenic responses of the his tail. Consequently, we made a new construct and removed the C-terminal hexahistidinetag from the scFvD9D10 (humanized scFvD9D10H6⁻). This was done by cutting vector pscFvD9D10V$_{Hum}$ with XhoI and EcoRI and substituting the His6-tail with a tandem stop codon and a unique Ncol site for easy identification. This was accomplished using two synthetic oligo's (oligo 1: 5'-

10　　　TCGAGATCAAACGGTAATAGCCATGG-3'; oligo 2: 5'-AATTCCATGGCTATTACCGTTTGATC-3') which, when annealed, reconstitute the D9D10 V$_L$ coding sequence, followed by tandem stop codons and a unique NcoI site for identification. The annealed double-stranded oligo has sticky ends corresponding to a XhoI site at the 5' end and EcoRI site at the 3' end. The oligo was ligated into the XhoI/EcoRI opened pscFvD9D10V$_{Hum}$

15　　　vector resulting in pscFvD9D10V$_{Hum}$[H6⁻]. Expression analysis showed identical expression levels and localisation compared to the His6-tagged D9D10 in *E. coli*.

2. Generation of humanized, chimeric D9D10

20　　　　Two fusion cDNA-genes respectively coding for the heavy and light chain fusion-proteins of the humanized D9D10 whole antibody were constructed. The light chain fusion cDNA consists of the cDNA encoding the mouse D9D10 light chain leader sequence (Ldr), needed for efficient transport of the fusion protein in the host cell, the humanized D9D10 light chain variable domain cDNA (V$_{Lh}$), followed by a human immunoglobulin kappa-light chain constant

25　　　domain (C$_L$).

The heavy chain fusion cDNA consists of the mouse D9D10 light chain leader cDNA-sequence (Ldr), followed by the humanized D9D10 heavy chain variable domain cDNA (V$_{Hh}$) and a human IgG1 heavy chain constant domain (C$_H$ = C$_H$1-Hinge-C$_H$2-C$_H$3) cDNA, in which the C1q-complement binding site in the C$_H$2 region, known to induce complement activation

30　　　upon injection of the recombinant antibody, was mutated (Pro$_{331}$–Ser) (Xu et al., 1994).

PCR cloning of human immunoglobulin Cγ1 and Cκ cDNA

Total RNA was isolated from human tonsil cells (frozen pellet of ±10⁷ cells) following the Chomczynski GuSCN/acid phenol isolation method (Chomczynski and Sacchi, 1987). 140µg
5   total RNA was obtained. cDNA was prepared by annealing 700 ng total RNA to 300 ng random hexamers (Pharmacia, Upsala, Sweden) and reverse transcription for 90 min at 42°C using AMV reverse transcriptase (RT-Stratagene) in a final volume of 20 µl (50 mM Tris pH 8.3, 40 mM KCl, 6 mM MgCl2, 5 mM DTT). The reaction was inactivated by heating at 90°C for 15 min.

10   *Cloning of the human Cκ cDNA :*

The cDNA was used as template for PCR amplification of the human Cκ cDNA using primer sequences based on the Genbank database sequence . accession # V00557 and # J00241.

oligo #7061 (Cκ sense primer):
15   *ThrValAla...*

5'-TCGAAGCTTAGTACTGTGGCTGCACCATCTGT-3'
         HindIII ScaI

oligo #7060 (Cκ antisense primer):
20   *CysGluGly...*

5'-GTCGAATTCTGCGCACTCTCCCCTGTTGAAGC-3'
         EcoRI FspI

PCR amplification using the 7060/7061 primers is expected to yield a fragment of 342
25   basepairs. ScaI / FspI digestion of this fragment should yield a blunt fragment starting at the first AA, Thr of Cκ and ending at the last AA, Cys. A stop codon is not present.

PCR reaction was carried out in a final volume of 50 µl, using 2µl of the RT reaction, 10 pmol of each primer and 5U of either Taq DNA polymerase (Stratagene, La Jolla, CA, USA). dNTPs were present at a final concentration of 200 µM in 1x Taq buffer as provided by the
30   supplier. Reactions were overlaid with 75 µl paraffin oil. Cycling conditions were as follows. After an initial denaturation of 5 min at 95°C 40 PCR cycles (1 min 94°C, 1 min at appropriate

annealing temperature of 60°C and 1 min at 72°C) were carried out. There was a final extension

phase of 10 min at 72°C. 5 µl amounts of the reaction were run on agarose gels.

  The PCR reaction with the 7060/7061 primer pair yielded a single band of ± 300 bases,

which was purified using the Geneclean™ kit (Bio101, Vista, CA, USA), digested with

5 EcoRI/HindIII, phenol:CHCl₃ extracted and ligated into EcoRI/HindIII digested pBSK(-) vector

(Stratagene). The ligation mix was electroporated into the DH5αF' bacterial strain. Transformed

bacteria were plated onto X-gal/IPTG LB agar plates for blue/ white selection of recombinants.

Four white colonies were selected for further analysis and plasmid DNA was prepared.

EcoRI/HindIII restriction analysis showed that all 4 Cκ transformants contained an insert of the

10 correct length. The 4 inserts were entirely sequenced . One clone was completely identical to the

database sequence (accession nrs V00557 and J00241). The corresponding plasmid was named

pBLSKIGkappaC.

     Cloning of the human Cγ1 heavy chain constant domain cDNA :

15 The cDNA was used as template for PCR amplification using primer sequences based

on the Genbank database sequence: accession # Z17370.

oligo #7601 (Cγ1 sense primer; 48-mer, should only be Cγ1 specific)

     AlaSerThr...

20 5'-CTA<u>GAATTC</u>T<u>GCGCA</u>TCCACCAAGGGCCCATCGGTCTTCCCCCTGGCA-3'

    EcoRI Fspl

oligo #7600 (Cγ1 antisense primer):

     LysGlyProSer...

25 5'-GTA<u>AAGCTT</u><u>GAGCTC</u>TTACCCGGAGACAGGGAGAGG-3'

    HindIII SacI

  PCR amplification using the 7601/7600 primer couple is expected to yield a fragment of

1016 basepairs. Fspl/SacI cleavage of this fragment followed by removal of the SacI 3' overhang

30 should yield a blunt fragment starting with the first AA, Ala of Cγ1 and ending with the last AA,

Lys. A stop codon is not included. PCR reactions were carried out in a final volume of 50 µl,

using 2μl of the RT reaction, 10 pmol of each primer and 5U of Taq DNA polymerase (Stratagene). dNTPs were present at a final concentration of 200 μM in 1x Taq buffer as provided by the supplier. Reactions were overlaid with 75 μl paraffin oil. Cycling conditions were as follows: after an initial denaturation of 5 min at 95°C 40 PCR cycles (1 min 94°C, 1 min

5   at appropriate annealing temp. 55°C and 1 min at 72°C) were carried out. There was a final extension phase of 10 min at 72°C. 10 μl amounts of the reaction were run on agarose gels. A single band of around 1kb was obtained. The 1kb band, obtained with the 7601/7600 primer pair, was purified using the Qiaquick ™-kit (Qiagen, Hilden, Germany) and ligated into pGEM-T- vector. The ligation mix was transformed into the DH5αF' bacterial strain. Transformed

10   bacteria were plated onto X-gal/IPTG LB agar plates for blue/ white selection of recombinants.

Eight white colonies were selected for further analysis and plasmid DNA was prepared. Restriction analysis with BstXI (= specific for IgG-1; absent in IgG-2) showed that 6 transformants contained an Cγ1 insert of the correct length. One clone was entirely sequenced and was shown to be identical to the database sequence, except for 3 codon switches, wich

15   correspond to a described allotypic variant Gm(-1,4) of the human IgG1 (lys214->arg214, asp356->glu356 and leu358->met358 respectively). Since the Gm(-1) ("nonmarker"), glu356/met358, also occurs on Cγ2, this marker will likely not be immunogenic when introduced in humans. The cloned sequence also contained two silent base switches in comparison to the database sequence Z17370. The final construct was named pGEMThIGG1c.

20   The C1q-complement binding site present in the C_H2 region of the human IgG1, known to induce complement activation upon injection of the recombinant antibody (Xu et al., 1994), was later mutated (Pro_{331}→Ser) as described further during the assembly of the humanized D9D10 fusion cDNA.

25   *Construction of fusion cDNAs*

In order to assemble the light- and heavy chain fusion genes, several intermediate cloning constructs, generated by PCR-assembly and amplification, were needed.

30   *Assembly of the light chain fusion cDNA*
The mouse D9D10 Vκ leader sequence cDNA was cloned by PCR-assembly (Stemmer

et al., 1995) of four partially overlapping synthetic oligonucleotides [IG8180, IG8179, IG8178 and IG8176] of each 40 bps, and subsequent PCR-amplification with two specific outside primers [IG 8175 and 8174]. The resulting 100 bp PCR fragment I, named Ldr, consist of a 5' untranslated region of 20 bp , including an XbaI cloning site, and the cDNA encoding the complete D9D10 Vκ leader peptide (20 AA) and 20 bp of the humanized D9D10 light chain variable domain cDNA encoding the first 6 AA.

Sense strand oligos :

<u>XbaI</u>

IG8180   5'-GTCCCCCGGGTACC<u>TCTAGA</u>ATGGATTTTCAAGTGCAGAT-3'

IG8179   5'-TTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATACTCTCG-3'

Antisense strand oligos :

IG8178   5'-CTCTGGGTCAGCTCGATGTCCGAGAGTATGACTGAGGCAC-3'

IG8176   5'-TGATTAGCAGGAAGCTGAAAATCTGCACTTGAAAATCCAT-3'

PCR amplification primers :

                                        XbaI

IG8175(sense)   5'-GTCCCCCGGGTACC<u>TCTAGA</u>ATG-3'

IG8174(antisense)   5'-CTCTGGGTCAGCTCGATGTCC-3'

IG8175→
        IG8180   IG8179

       --------------------   ------------------
                   IG8176   IG8178

       --------------------   ------------------
                                        IG8174←

42

The humanised light chain variable domain as present in pGEM-T-V$_L$H, described earlier, was PCR-amplified using primers [IG8172 and IG8171] designed to produce PCR fragment II containing the complete variable domain cDNA with exception of the last 3 aminoacids (IKR), and flanked at the 3'-terminus by an XhoI-cloning site.

5

IG8172(sense)   5'-GACATCGAGCTGACCCAGAGCCCGGCG-3'

                                          XhoI
IG8171(antisense)   5'-CGCGCTCGAGTTTGGTACCCTG-3'

10

Fusion of the two DNA fragments PCR-I (Ldr) and PCR-II (V$_{Lh}$), having 20 bp overlap, was performed by overlap PCR using primerset IG8175 and IG8171. The resulting PCR-III fragment was directly cloned in pGEM-T resulting in the pGEMLdrV$_{Lh}$ plasmid.

15   XbaI
IG8175 (sense)   5'-GTCCCCCGGGTACCTCTAGAATG-3'

                                          XhoI
IG8171 (antisense)   5'-CGCGCTCGAGTTTGGTACCCTG-3'

20

The human κ-light chain constant domain was cloned by PCR-amplification using pBLSKIGkappaC as template with primers IG8170 and IG8169. The resulting PCR-IV fragment consists of the cDNA sequence encoding the last 3 AA of V$_{Lh}$ and the complete human Ckappa constant domain, followed by a stop codon and an EcoRI cloning site. The PCR-IV DNA was

25   directly cloned in the pGEM-T vector resulting in the pGEM-TC$_L$ plasmid.

                                          XhoI
IG8170(sense)   5'-GCGCCTCGAGATCAAACGGACTGTGGCTGCACCATCTG-3'

                                          EcoRI
30   IG8169(antisense)   5'-GCCGGAATTCCTAGCACTCTCCCCTGTTGAAG-3'

43

Fusion of LdrV$_{Lh}$ and C$_L$ cDNA in the pGEM-T backbone was realised by insertion of the C$_L$- containing XhoI-SpeI fragment, isolated from pGEM -TC$_L$ plasmid, in the pGEMLdrV$_{Lh}$ plasmid. The resulting construct was named pGEMhD9D10$_L$.

5   *Assembly of the heavy chain fusion cDNA*

The mouse D9D10 Vκ leader sequence cDNA was cloned by PCR-assembly (Stemmer et al., 1995) of four partially overlapping synthetic oligonucleotides [IG8180, IG8179,IG8176 and IG8177] of each 40 bps, and subsequent PCR-amplification with two specific outside primers [IG 8175 and 8173]. The resulting 100 bp PCR-V fragment, named Ldr-2, consist of a

10   5' untranslated region of 20 bp, including an XbaI cloning site, and the cDNA encoding the complete D9D10 Vκ leader peptide (20 AA) and 20 bp of the humanized D9D10 heavy chain variable domain cDNA encoding the first 6 AA.

Sense strand oligos :

15   XbaI
      IG8180   5'-GTCCCCCGGGTACC<u>TCTAGA</u>ATGGATTTTCAAGTGCAGAT-3'

      IG8179   5'-TTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATACTCTCG-3'

20   Antisense strand oligos :

      IG8177   5'-CTCTGCACCAGCTGCACCTGCGAGAGTATGACTGAGGCAC-3'

      IG8176   5'-TGATTAGCAGGAAGCTGAAAATCTGCACTTGAAAATCCAT-3'

25

      PCR amplification primers :

                                            XbaI
      IG8175(sense)   5'-GTCCCCCGGGTACC<u>TCTAGA</u>ATG-3'

30   IG8173(antisense) 5'-CTCTGCACCAGCTGCACCTGC-3'

```
IG8175→
        IG8180                      IG8179

    ------------------  ------------------
              IG8176          IG8177
```
5
```
              ------------------  ------------------
                                      IG8173→
```

The humanised variable heavy chain domain as present in pGEM-T-V$_H$H, described
earlier, was PCR-amplified using primers (IG8168 and IG8167) designed to produce PCR-VI
10 fragment containing the complete variable domain cDNA, and flanked at the 3'-terminus by an
XhoI-cloning site.

IG8168(sense)   5'-CAGGTGCAGCTGGTGCAGAGCGGTAG-3'

15 IG8167(antisense)

               XhoI
    5'-CGCCGGCTCGAGACGGTGACCGTGGTCCCTTGGCCCCAGTAATCC-3'

Fusion of Ldr-2 and V$_{Hh}$ was performed by overlap PCR on a mixture of PCR-V and
20 PCR-VI using sense primer IG 8175 and an antisense primer IG 8166, resulted in a PCR
fragment (LdrV$_{Hh}$) which was directly cloned in a pGEM-T vector , resulting in pGEMLdrV$_{Hh}$.

                                    XbaI
    IG8175(sense)   5'-GTCCCCCGGGTACCTCTAGAATG-3'
25

                        XhoI
    IG8166(antisense)   5'-CGCCGGCTCGAGACGGTGACC-3'

The human heavy chain constant domain cDNA was produced by PCR amplification on
30 pGEMThIGG1c as template, using sense primer IG 8165, designed to introduce a XhoI
restriction site and antisense primer IG 8164 that added an extra leucine to the C$_H$ sequence and
introduced a STOP codon followed by an EcoRI cloning site. The introduction of a codon for

a leucine provided, together with the codon for a lysine (normally the last aminoacid), a HindIII restriction site. This HindIII site was used to insert a scFv-module (cfr MoTAbII expression plasmids, see below). The resulting fragment PCR-VII was inserted in the pGEM-T vector resulting in plasmid pGEM-TC$_H$.

5

```
                                      XhoI
IG8165(sense)              5'-GCCGCTCGAGCGCATCCACCAAGGGC-3'
```

```
                                      EcoRI       HindIII
10    IG8164(antisense)       5'-GCCGGAATTCGCTAAAGCTTACCCGGAGACAGGGAGAGG-3'
```

The amino acid Pro at position 331 in the C$_H$2 domain of both IgG1 and IgG4 immunoglobulins is described to contribute to their differential ability to bind and activate complement (Xu et al., 1994). The Pro331-codon CCC was therefore mutated to a Ser331-codon, TCC. Two specific primers IG 8460 and IG8459 were designed, to introduce this mutation by PCR mutagenesis.

Two separate PCR-amplifications were performed on pGEM-T-C$_H$ as template using (1) primers IG2617, matching with the T7-promoter region in pGEM-T and IG8460, resulting in a 733 bp PCR-VIII fragment, and (2) primers IG 8459 and IG3899, matching the SP6-promoter in pGEM-T, resulting in a 473bp PCR-IX fragment. Overlap PCR was subsequently performed on a mixture of PCR-VIII and PCR-IX, using again the primers IG2617 and IG3899, resulting in a 1178 bp PCR-X fragment. The amplified PCR-X fragment was eventually inserted as an XhoI-SpeI fragment (1018 bp) in the pGEMLdrV$_{Hh}$ plasmid. The resulting pGEMhD9D10$_H$ plasmid contains the complete coding sequence of the humanized D9D10 heavy chain fusion protein.

```
IG8459 (sense)              5'-GCCCTCCCAGCCTCCATCGAGAAAAC-3'
                                                 Ser331
IG8460 (antisense)          5'-GTTTTCTCGATGGAGGCTGGGAGGGC-3'
30                                               Ser331
```

IG2617 (sense-T7)   5'-TAATACGACTCACTA-3'

IG3899 (antisense-SP6)   5'-ATTTAGGTGACACTATAG-3'

5   *Construction of mammalian expression plasmids*

Successful high level expression of recombinant immunoglobulins has been reported in both lymphoid and non-lymphoid mammalian cell lines. Basically an expression plasmid(s), containing the immunoglobulin genes coding for respectively heavy and light chain proteins

10   under transcriptional control of a promoter/enhancer unit recognized in mammalian cells, is introduced in the chosen host cells together with (as one plasmid or on separate plasmids) a drug-resistance gene expression unit by classical cell transfection techniques. Cells that have randomly integrated the foreign expression units in their cell genome are intially selected for their drug-resistant phenotype and secondly for high level, stable expression of the protein of

15   interest, the immunoglobulin. After gene integration, an increase in the immunoglobulin expression level can be obtained by coamplification of the genes through further selection of isolated recombinant cell lines for increased resistance to the drug resistance marker.

One possible example of a successful strategy for mammalian cell expression is the glutamine synthetase based selection/amplification method shown to result in high level

20   production of mammalian proteins in different cell types including Chinese hamster ovary cells (CHO) (Cockett et al., 1990) and myeloma cells, Ns0 (Bebbington et al. , 1992). The use of the system is covered by patents WO87/04462 and WO89/10404 (Lonza Biologicals, Slough, UK).

Following the GS-expression method, the fusion genes coding for respectively the heavy- and light chain of the recombinant immunoglobulins were cloned in a mammalian expression

25   plasmid (pEE12 or pEE14) under transcriptional control of the strong Cytomegalovirus major immediate early promoter /enhancer (CMV-MIE). This plasmid also carries a cloned glutamine synthetase (GS) gene expression element that can act as a dominant selectable marker in a variety of cells. GS indeed provides the only pathway for synthesis of glutamine using glutamate and ammonia as substrates. The final fusion product $LdrV_{Lh}C_L$ or $hD9D10_L$ was directly cloned

30   as an XbaI-EcoRI fragment isolated from the plasmid $pGEMhD9D10_L$ in the mammalian expression vectors pEE14 (for CHO) and pEE12 (for Ns0) (Lonza biologicals) under

transcriptional control of the CMV promoter, resulting in the plasmids pEE12hD9D10$_L$ and pEE14hD9D10$_L$.

The cDNA encoding the heavy chain fusion protein LdrV$_{Hh}$C$_H$ or hD9D10$_H$ was first transferred from the pGEMhD9D10$_H$ construct as an XbaI-EcoRI fragment in the intermediate

5   vector pEE6hCMV-BglII (Lonza Biologicals), also behind the CMV promoter. From the latter construct pEE6hD9D10$_H$ a complete mammalian expression casette, consisting of CMV-promoter followed by the fusion gene and a polyadenylation site, were transferred as an BglII-BamHI DNA fragment in the BamHI opened plasmids pEE12hD9D10$_L$ and pEE14hD9D10$_L$ expression plasmids already available. The final expression plasmids, named pEE12hD9D10

10   and pEE14hD9D10 then consists of the pEE-backbone plasmid containing the GS-selection unit, carrying the light chain fusion gene expression casette followed by a comparable heavy chain fusion gene expression casette.

The approach of assembling a single expression plasmid containing separate transcription units for both heavy and light chains and the selectable marker, is adviced in order to ensure

15   coamplification with the marker gene.

A schematic representation of both plasmids is given in figures 5 and 6.

The cDNA sequence encoding the complete humanized D9D10 heavy chain fusion protein is given in figure 7.

The cDNA sequence encoding the humanized D9D10 light chain fusion protein is given

20   in figure 8.

The amino acid sequence of the humanized D9D10 heavy chain fusion protein is given in figure 9.

The aminoacid sequence of the humanized D9D10 light chain fusion protein is given in figure 10.

25

Small scale expression of humanized D9D10 chimeric antibody in COS cells

A quick way to determine the feasibility of expressing a recombinant protein in mammalian cells and to evaluate its functionallity, is transient expression of the product in COS

30   cells (Gluzmann, 1981). COS cells are Simian Virus 40 (SV40)-permissive CV1 cells (African monkey kidney) stably transformed with an origin-defective SV40 genome, thereby

constitutively producing the SV40 T-antigen. In SV40-permissive cells, T-antigen initiates high copy number transient episomal replication of any DNA-vector that contains the SV40 origin of DNA replication. Both the pEE12 and pEE14 expression vectors contain an SV40 origin of replication in the SV40 early promoter region controlling the GS-selection gene, and thus permits efficient transient expression in COS cells.

Small amounts of functionally active antibody were made by transient expression in COS cells. COS7 cells (ATCC CRL 1651) were routinely cultured in DMEM supplemented with 0,03% glutamine and 10% fetal calf serum. For preparative scale transfection, an optimized DEAE-transfection protocol (McCutchan, 1968) was used. Alternatively, other well known transfection methods such as Ca-phosphate precipitation, electroporation, liposome-based transfection can be used. Briefly, exponentially growing COS7 cells were seeded in cell factories (Nunc, Rochester, NY, USA) at 3.5 10^4 cells/cm^2 about 18 h before transfection, after which the cells were washed twice with MEM-Hepes pH 7.1 (Gibco, Rockville, MD, USA) and allowed to cool to bench temperature. 0,5 µg/cm^2 cell surface of high quality plasmid DNA (CsCl-density purification) of the mammalian expression plasmids pEE12hD9D10 and pEE14hD9D10 was ethanol precipitated, redissolved in 25 µl/cm^2 MEM-Hepes pH 7.1 and slowly added to the same volume of 2 mg/ml DEAE-dextran MW 500.000 (Pharmacia) in MEM-Hepes pH 7.1. The DNA-DEAE-dextran precipitate (50 µl/cm^2) was allowed to form for 20-25 min, put on the cells for 25 min and removed to be stored at -20°C (the same precipitate can be reused in a second transfection experiment with the same efficiency).

The cells were incubated during the next 3.5 hours in DMEM growth medium (Gibco) containing 0.1 mM chloroquine (Sigma) (0,3ml /cm^2) in a CO$_2$-incubator at 37°C, then washed two times with growth medium and further incubated for 18 hrs in complete culture medium enriched with 0.1 mM sodium butyrate (Sigma) at 37°C (0,3ml /cm^2). The next day the cells were washed twice with serum free DMEM medium supplemented with 0.03% glutamine (Merck) and then incubated for 48h (determined in analytical scale experiments as the optimal harvest time) in 150 µl/cm^2 cell surface of the same medium at 37°C, after which conditioned medium was harvested and stored at -70°C untill purification. As negative control COS cells were also transfected with the empty expression vectors pEE12 and pEE14.

Quality control of the crude CM was performed by IFNγ-binding assay in ELISA format, by SPR-analysis and by measuring the inhibition of IFNγ mediated MHC class II-induction.

Human Interferon γ-coating Elisa

96 well ELISA culture plates (Nunc 469914) were coated with 100 ng/well hIFNγ (Genzyme 80-3348-01, 1mg/ml) diluted in 50mM TrisHCl pH8.5, 150 mM NaCl, by 18h incubation at 4°C. Blocking of nonspecific binding was performed in PBS/0.1% caseine
5   (200µl/well, 1h ,37°C). All washing steps were performed with PBS/0.05% Tween-20 (3 x 200µl/well). Purified mouse-human chimeric D9D10 whole antibody (EP 0 528 469 to Billiau and Froyen), produced by transient expression in COS cells, was used as positive control (concentration range 500ng/well to 4 ng/well, ½ dilution series prepared in the sample diluent, 100µl/well). Samples were diluted in a ½ dilution series in PBS/0.1 % caseine, and incubated
10   for 2h at 37°C. Detection was performed using an alkaline-phophatase conjugated goat-anti-human IgG$_{H+L}$ (PromegaS3821), diluted 1/2000 in PBS/0.05% caseine, incubated for 2h at 37°C. AP-substrate (SigmaN-2765) was used at a concentration of 1mg/ml in 100mM TrisCl pH9.5, 100mM NaCl, 5mM MgCl$_2$. Plates were analysed at 405/595 nm after resp. 15 and 30 min incubation at 37°C.

15   Results are shown in figure 11 : humanized D9D10 clearly interacts with human IFNγ coated onto the wells.

SPR analysis

A comparable set up was used as described for the evaluation of the murine and
20   humanized scFvD9D10 derivatives. Briefly, murine D9D10 was immobilized directly onto a B1 sensorchip (BIACORE AB) - containing less carboxylic groups and for which as such no pretreatment is necessary - at a concentration of 10 µg/ml D9D10 in an acetate buffer pH 4.8 using amine coupling. A fixed concentration of 8 µg/ml human IFNγ was added, followed by the injection of either murine D9D10 (10 µg/ml; positive control) or crude COS supernatant
25   containing humanized D9D10. Results are shown in figure 12. These data clearly illustrate the presence of active, IFNγ binding molecules in the COS supernatant. As no exact concentrations were determined of the humanized D9D10, no affinity data were calculated.

Inhibition of MHC class II-induction
30   see example 8.1.

Purification of humanized D9D10

Humanized D9D10 was purified using classical protein A chromatography (Perry and Kirby, 1990; Page and Thorpe, 1996). Quality control of the purified antibody construct was performed by Western Blot (classical technology) and ELISA. The latter is done as described above and results are shown in figure 13. From these results it is clear that purified, humanized D9D10 is specifically interacting with IFNγ coated onto the wells.

Generation of stable mammalian expression cell lines

For generation of stable mammalian expression cell line, two host cell lines Ns0 (Galfre and Milstein, 1981; ECACC 85110503) and CHO-K1 (ATCC CCL61) were used.

The glutamine-dependent NS0 cells were routinely cultured in Lonza DME (JRH 51435)/200mMglutamine/10%FCS. High quality plasmid DNA pEE12hD9D10, prepared by CsCl-density purification, and linearized by SalI digestion, was used for transfection of the NS0 cells by electroporation (40 µg DNA/ 10^7 cells). Transfected cells were then selected for the glutamine-independent phenotype by gradual reducing the glutamine concentration. Selection was performed in Lonza DME (JRH51435)/GS supplement (JRH58672)/10% dialysed FCS. Individual NS0 clones were isolated after ± 2 weeks of selection. The clones were analysed for recombinant antibody production and secretion by testing the cell conditioned medium in the IFNγ-coating ELISA described earlier.

Several positive cell lines were selected for subsequent vector amplification by growth in the presence of the GS-inhibitor MSX (methionine sulfoximine), resulting in increased humanized D9D10 antibody expression levels.

Large scale production of the recombinant antibody using high expressing NS0 recombinant cell lines is done in bioreactor systems (e.g. hollow fibre systems)

CHO-K1 cells were routinely cultured in GMEM-S (JRH51492)/200mM glutamine/10%FCS. High quality plasmid DNA pEE14hD9D10, prepared by CsCl-density purification, was directly used for transfection of CHO-K1 cells by Ca^{2+}-phosphate transfection

techniques ($12\mu g/1.15\ 10^6$ cells seeded 18h before transfection on T-flasks). Selective medium, GMEM-S(JR51492)/GS supplement(JRH58672)/10%dialysed FCS/$25\mu M$ MSX was added to the cells 24h post-transfection. Individual clones could be isolated ± 2 weeks after transfection. Selected clones were analysed for recombinant antibody expression and secretion by testing the

5   cell conditioned medium in the IFNγ-coating Elisa described earlier.   Several positive cell lines were selected for subsequent vector amplification by growth in the presence of increased concentrations of the GS-inhibitor MSX, resulting in increased antibody expression levels.

Large scale production of the recombinant antibody using high expressing CHO-K1 recombinant cell lines is done in bioreactor systems (e.g. hollow fibre or ceramic core systems)

10

3. Generation of humanized sheep anti-IFNγ antibodies

Sheep antibodies were generated by immunizing sheeps according to standard immunization protocols. Briefly, sheeps were injected intradermally on multiple sites with the

15   antigen (recombinant human IFNγ(procaryotic origin)) for several times over a timeframe of several months (day 0, 14, 28, 56, extra injections on a monthly basis). Serum is tested for its antiviral activity and its affinity (using SPR analysis).

As elution conditions necessary to elute an antigen from its antibody reflect the affinity of the antibody (McCloskey et al., 1997), experiments are performed in which the elution

20   conditions of the sheep antibodies for human IFNγ were compared with those of the scFvD9D10 antibody.

Sheep monoclonal antibodies are generated by fusing B-lymphocytes isolated from peripheral blood with murine Sp2/0 myeloma cells according to the protocol as described in example 1. The affinity of the antibodies for human IFNγ is determined by SPR analysis as

25   described in example 1.

4. Generation of anti-IFNγ tetravalent antibody constructs

4.1. Generation of MoTAb I

30

The MoTAb I (Monospecific Tetravalent Antibody) molecule is defined as a molecule

which consists of 4 identical scFv molecules (e.g. humanized D9D10 scFv's) in the format of a homodimer of two identical molecules, each containing two scFv's. Both scFv's are linked together using a dimerisation domain, which drives the homodimerisation of the molecule (see figure 1). Comparable structures have already been described (Pack et al., 1995, Plückthun &

5   Pack, 1997).

The humanized D9D10 scFv was used as a building block to generate the MoTAbI molecule using standard recombinant DNA techniques. A single MoTAb subunit started with a humanized D9D10 scFv followed by a dimerisation domain flanked by flexible linkers. The dimerisation domain was in turn linked C-terminally to a second D9D10 scFv. Finally a

10   detection and purification tag was added to the extreme C-terminus of the molecule. However, in order to circumvent possible immunological reactions against the tag, MoTAb I was also produced in an untagged version. The sequence coding for the dimerisation domain and the flanking linkers were made synthetically using the method described by Stemmer et al. (1995). This synthetic domain was subsequently linked to both D9D10 scFv's. As linkers between the

15   dimerisation domain and the scFv's, we have used the flexible and proteolysis-resistant truncated human IgG3 upper hinge region (Pack & Plückthun, 1992). As dimerisation domain we used either the helix-turn-helix motif described by Pack *et al.* (1993) or the leucine-zipper dimerisation domain originating from the human JEM-1 protein as described by Duprez et al. (1997). Optionally, an additional cysteine residue is inserted next to the dimerisation domain

20   to provide extra stability. When applicable, a C-terminal detection and purification tag e.g. a hexahistidine sequence, is used. The sequences were assembled in such a way that functional domains were easily replaceable using unique restriction sites present in the molecule. For the construction of the pGEM-THDH vector, we synthesized 10 oligo's which collectively encode both strands of the HDH region (hinge region-dimerization domain-hinge region) flanked by a

25   XhoI and a SpeI restriction site. The plus strand as well as the minus strand consist of 5 oligo's configured in such a way that, upon assembly, complimentary oligo's will overlap by 20 nucleotides. In these oligo's the codons where optimised for optimal *E.coli* usage. The resulting 223 bp fragment was cloned into a pGEM-T vector and several clones were sequenced.

30

Assembly oligonucleotides for the HDH-domain:

Oligo No.	Oligo Seq.
1s	5'-CGCGCTCGAGATCAAACGGACCCCGCTGGGTGATACCACTC-3'
2as	5'-CAGTTCACCTCCGGAGGTATGAGTGGTATCACCCAGCGGG-3'
3s	5'-ATACCTCCGGAGGTGAACTGGAAGAGCTGTTGAAACATCT-3'
4as	5'-GACCTTTCAGCAGTTCTTTCAGATGTTTCAACAGCTCTTC-3'
5s	5'-GAAAGAACTGCTGAAAGGTCCGCGGAAAGGTGAACTGGAG-3'
6as	5'-TTCAGGTGCTTCAGCAATTCCTCCAGTTCACCTTTCCGCG-3'
7s	5'-GAATTGCTGAAGCACCTGAAAGAGCTGTTGAAAGGTACCC-3'
8as	5'-ATGGGTAGTATCACCTAGGGGGGGTACCTTTCAACAGCTCT-3'
9s	5'-CCCTAGGTGATACTACCCATACCAGCGGTCAGGTGCAACT-3'
10as	5'-CGCGGAATTCGCGTTCGCGACTAGTTGCACCTGACCGCTGGT-3'

Amplification oligonucleotides for the HDH-domain:

Oligo No.	Oligo Seq.
1s	5'-CGCGGTATACTGACCCAGAGC-3'
2as	5'-CGCGCTCGAGTTTGGTACCCTG-3'

The MoTAbI expressionplasmid was constructed as followed: The scFvD9D10 coding sequence was amplified by PCR using the pscFvD9D10V$_{Hum}$ plasmid as a template. The sense primer used in this amplification carried a unique SpeI restriction site in such a way that the resulting scFvD9D10 sequence could be fused in-frame at the C-terminus of the dimerisation domain.

sense primer:	5'-CGCGACTAGTGCAGAGCGGTAGCGAACTG-3'
antisense primer:	5'-GCCAGTGAATTCTATTAGTGGTGATG-3'

The resulting PCR fragment was inserted into the pGEM-T vector and verified by DNA sequence analysis. The resulting plasmid was named pGEM-TscFvD9D10 f s/e. Subsequently, the MoTABI expressionplamid was assembled in a three-point ligation using following fragments: The N-terminal scFvD9D10 originating from vector pscFvD9D10V$_{hum}$ as a

54

XhoI/EcoRI fragment. This fragment also carried the antibiotic resistance gene (Amp), the origin of replication and the expression- and secretion signals. A second fragment, originating from pGEM-THDH cut with XhoI and SpeI, carried the helix-turn-helix dimerisation domain already described previously flanked by human IgG3 upper hinge regions. Finally, a third

5   fragment, originating from the SpeI/EcoRI cut pGEM-TscFvD9D10 f s/e plasmid, carried the C-terminal scFvD9D10 with the hexahistidine tag. The final expressionplasmid was named pMoTAbIH6 (Figure 14) and carried the MoTAbI molecule under control of the lac promotor and the pelB signal sequence as the secretion signal (Figure 15 and 16).

To reduce immunogenicity, the hexahistidine sequence was removed using synthetic oligo's in

10   a similar way as described previously for the humanized scFvD9D10, resulting in MoTabI.

The MoTAb I expression plasmid was introduced into a suitable *E.coli* expression strain, e.g . JM83 and BL21. Good expressionlevels could be obtained in both strains. Detection of the MoTabI molecule (60 kDa) on western blot was done with an anti D9D10 rabbit polyclonal antibody and/or an anti His6 monoclonal antibody (Babco). However, only a minor amount of

15   the MoTAbI molecule was present in a soluble form in the bacterial periplasm. The majority of the MoTAbI molecule was not able to traverse the bacterial membrane and was present as cytoplasmic inclusion bodies. This was confirmed by N-terminal amino acid sequencing which revealed still the presence of the pelB signal sequence on the molecule. The functionality of the minor amount of secreted MoTAbI could however be confirmed using an ELISA. In this

20   ELISA, recombinant human IFNγ was coated onto a polystyreneplate and incubated with periplasmic fractions originating from *E.coli* cells expressing the MoTAbI molecule. Bound MoTAbI molecules where then detected using a rabbit polyclonal serum generated against the D9D10 scFv followed by a peroxidase labeled goat anti rabbit secondary serum.

Since most MoTAbI molecules were present in cytoplasmic inclusion bodies, the molecules were

25   purified from this fraction under denaturing conditions followed by refolding to functional molecules. However, since the MoTAbI molecule has the pelB signal sequence still attached, a new cytoplasmic expressionplasmid was constructed. In this expressionplasmid, MoTAbI expression is under control of the strong leftward promotor of phage lambda (P_L). Since no secretion to the periplasmic space is necessary, the MoTAbI coding sequence was fused directly

30   to an ATG startcodon. This was accomplished by isolating the MoTAbI coding sequence lacking the pelB signal sequence by PCR from the pMoTAbI expressionplasmid and recloning

it into the EcoRV opened pBSK(+) vector (Stratagene). A SapI restriction site giving access to
the first mature codon was hereby generated. After DNA sequence verification the MoTAbI
coding sequence was inserted as a SapI blunt/ SalI fragment into the NcoI blunt/ SalI cut pIGRI2
vector.

5

* pIGRI2 expressionvector nucleotide sequence


```
   1   TTCCGGGGATCTCTCACCTACCAAACAATGCCCCCCTGCAAAAAATAAAT
  51   TCATATAAAAAACATACAGATAACCATCTGCGGTGATAAATTATCTCTGG
 101   CGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACATCAGCAGGA
 151   CGCACTGACCACCATGAAGGTGACGCTCTTAAAAATTAAGCCCTGAAGAA
 201   GGGCAGGGGTACCAGGAGGTTTAAATCATGGTAAGATCAAGTAGTCAAAA
 251   TTCGAGTGACAAGCCTGTAGCCCACGTCGTAGCAAACCACCAAGTGGAGG
 301   AGCAGTAACCATGGTTACTGGAGAAGGGGGACCAACTCAGCGCTGAGGTC
 351   AATCTGCCCAAGTCTAGAGTCGACCTGCAGCCCAAGCTTGGCTGTTTTGG
 401   CGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAA
 451   GCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCA
 501   CCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAG
 551   TGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAA
 601   CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTC
 651   GGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACG
 701   TTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACT
 751   GCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGC
 801   GTTTCTACAAACTCTTTTGTTTATTTTTCTAAATACATTCAAATATGTAT
 851   CCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATAAAAGGATCT
 901   AGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAG
 951   TTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTC
1001   TTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAAC
1051   CACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTT
1101   TTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCT
1151   TCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGC
```

1201 CTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGC

1251 GATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAA

1301 GGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGG

1351 AGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGAGAA

1401 AGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGG

1451 CAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCT

1501 GGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGA

1551 TTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAA

1601 CGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGT

1651 TCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTT

1701 GAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTC

1751 AGTGAGCGAGGAAGCGGAAGAGCGCTGACTTCCGCGTTTCCAGACTTTAC

1801 GAAACACGGAAACCGAAGACCATTCATGTTGTTGCTCAGGTCGCAGACGT

1851 TTTGCAGCAGCAGTCGCTTCACGTTCGCTCGCGTATCGGTGATTCATTCT

1901 GCTAACCAGTAAGGCAACCCCGCCAGCCTAGCCGGGTCCTCAACGACAGG

1951 AGCACGATCATGCGCACCCGTGGCCAGGACCCAACGCTGCCCGAGATGCG

2001 CCGCGTGCGGCTGCTGGAGATGGCGGACGCGATGGATATGTTCTGCCAAG

2051 GGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATTGATTGGCTCCAATT

2101 CTTGGAGTGGTGAATCCGTTAGCGAGGTGCCGCCGGCTTCCATTCAGGTC

2151 GAGGTGGCCCGGCTCCATGCACCGCGACGCAACGCGGGGAGGCAGACAAG

2201 GTATAGGGCGGCGCCTACAATCCATGCCAACCCGTTCCATGTGCTCGCCG

2251 AGGCGGCATAAATCGCCGTGACGATCAGCGGTCCAGTGATCGAAGTTAGG

2301 CTGGTAAGAGCCGCGAGCGATCCTTGAAGCTGTCCCTGATGGTCGTCATC

2351 TACCTGCCTGGACAGCATGGCCTGCAACGCGGGCATCCCGATGCCGCCGG

2401 AAGCGAGAAGAATCATAATGGGGAAGGCCATCCAGCCTCGCGTCGCGAAC

2451 GCCAGCAAGACGTAGCCCAGCGCGTCGGCCGCCATGCCGGCGATAATGGC

2501 CTGCTTCTCGCCGAAACGTTTGGTGGCGGGACCAGTGACGAAGGCTTGAG

2551 CGAGGGCGTGCAAGATTCCGAATACCGCAAGCGACAGGCCGATCATCGTC

2601 GCGCTCCAGCGAAAGCGGTCCTCGCCGAAAATGACCCAGAGCGCTGCCGG

2651 CACCTGTCCTACGAGTTGCATGATAAAGAAGACAGTCATAAGTGCGGCGA

2701 CGATAGTCATGCCCCGCGCCCACCGGAAGGAGCTGACTGGGTTGAAGGCT

2751 CTCAAGGGCATCGGTCGGCGCTCTCCCTTATGCGACTCCTGCATTAGGAA

2801 GCAGCCCAGTAGTAGGTTGAGGCCGTTGAGCACCGCCGCCGCAAGGAATG

2851 GTGCATGTAAGGAGATGGCGCCCAACAGTCCCCCGGCCACGGGGCCTGCC

2901 ACCATACCCACGCCGAAACAAGCGCTCATGAGCCCGAAGTGGCGAGCCCG

2951 ATCTTCCCCATCGGTGATGTCGGCGATATAGGCGCCAGCAACCGCACCTG

3001 TGGCGCCGGTGATGCCGGCCACGATGCGTCCGGCGTAGAGAATCCACAGG

3051 ACGGGTGTGGTCGCCATGATCGCGTAGTCGATAGTGGCTCCAAGTAGCGA

3101 AGCGAGCAGGACTGGGCGGCGGCCAAAGCGGTCGGACAGTGCTCCGAGAA

3151 CGGGTGCGCATAGAAATTGCATCAACGCATATAGCGCTAGCAGCACGCCA

3201 TAGTGACTGGCGATGCTGTCGGAATGGACGATATCCCGCAAGAGGCCCGG

3251 CAGTACCGGCATAACCAAGCCTATGCCTACAGCATCCAGGGTGACGGTGC

3301 CGAGGATGACGATGAGCGCATTGTTAGATTTCATACACGGTGCCTGACTG

3351 CGTTAGCAATTTAACTGTGATAAACTACCGCATTAAAGCTAATCGATGAT

3401 AAGCTGTCAAACATGAGAATTAA

The new vector is called pIGRI2MoTAbI. A version lacking the hexahistidine tag was constructed in a similar way starting from the previous MoTAbI expressionplasmid without hexahistidine tail. The new MoTAbI expressionvectors were subsequently transferred to *E.coli* expressionstrains MC1061(pAcI), SG4044(pcI857) and UT5600(pAcI). As expected, most of the expressed MoTAbI was present as cytoplasmic inclusionbodies. MoTAbI molecules were purified from cytoplasmic inclusion bodies under denaturing conditions followed by standard refolding procedures as described by De Bernardez Clark (1998).

4.2. Generation of MoTAb II

The D9D10 MoTAb II is defined as a humanized D9D10 whole antibody molecule to which a humanized D9D10ScFv sequence was attached at the carboxyterminus (CH3-domain) of the heavy chain (see Figure 1). A comparable type of molecule has already been described in literature (Coloma and Morrison, 1997).

For the expression of the D9D10 MoTAbII protein two fusion genes, respectively coding for heavy and light chain protein of the assembled antibody, were constructed. The heavy chain

fusion gene consists of an immunoglobulin leader sequence (D9D10 Vκ leader cDNA) followed

by the humanized D9D10 heavy chain variable domain cDNA, a human IgG1 heavy chain

constant domain (C_H1-Hinge-C_H2-C_H3) cDNA, a short G_3S linker sequence (Coloma and

Morrison, 1997) and the humanized D9D10 ScFv sequence. Alternative linker sequences such

5   as the $(G_4\ S)_3$ sequence or the flexible and proteolysis-resistant truncated mouse IgG3 upper

hinge region (Pack & Plückthun, 1992) can be used.

The light chain fusion gene is identical to the humanized D9D10 recombinant antibody

light chain gene (2) and contains the D9D10 Vκ leader, the humanized light chain variable

domain cDNA and the human IgG1 constant domain (kappa).

10

Construction of MoTAb II heavy chain cDNA

The basic constructs generated for expression of the humanized D9D10 antibody could

be used as backbone for the MoTAbII constructs. As described several intermediate cloning

15   constructs, mainly generated by PCR-assembly and -amplification, eventually resulted in two

final constructs, named $pGEMhD9D10_L$ and $pGEMhD9D10_H$. The latter plasmid was used as

acceptorfragment after digestion with HindIII and EcoRI, which eliminates the STOP codon for

insertion of a HindIII-EcoRI donorfragment isolated from a plasmid pGEM-T-D9D10HE,

resulting in the in frame fusion of the $hD9D10_H$ cDNA to a cDNA sequence encoding the

20   Gly_3Ser linker followed by the humanizedScFv-module and a STOP codon. The resulting

plasmid was named $pGEM\text{-}MoTAbII_H$.

pGEM-T-D9D10HE was constructed by PCR amplification using $pScFvD9D10V_{hum}$ as template

with primers IG8078 and IG8077. The resulting 755bp PCR fragment, containing the Gly_3Ser

linker followed by the humanized scFv-module and a STOP codon, was directly cloned in the

25   pGEM-T vector.

```
                           HindIII
IG8078 (sense):    5'-CCCAAGCTTGGCGGAGGCTCACAGGTGCAGCTGGTGCAGAG-3'
                           EcoRI
IG8077 (antisense): 5'-CGGAATTCTACCGTTTGATCTCGAGTTTGG-3'
```

Construction of mammalian expression plasmids

Expression in mammalian cell lines was performed completely as described for the humanized D9D10 antibody (cf example 2). The cDNA encoding the LdrV$_{Hh}$C$_H$ScFv or

5   MoTAbII$_H$ fusion protein was initially inserted in the pEE6hCMV-BglII (Lonza biologicals) intermediate expression vector, under transcriptional control of the hCMV promoter. This was performed by transfer of the EcoRI-XbaI DNA insert from pGEMMoTAbII$_H$ into the pEE6hCMV-BglII vector. From the pEE6MoTAbII$_H$ plasmid a complete mammalian expression casette, consisting of CMV-promoter followed by the fusion gene and a polyadenylation site,

10   was then transferred as a BglII/BamHI fragment to the BamHI opened pEE12hD9D10$_L$ and pEE14hD9D10$_L$ expression plasmids already available (construct was earlier described for the humanized D9D10 antibody construct in example 2). The final expression plasmids, named pEE12MoTAbII and pEE14MoTAbII then consisted of the pEE-backbone plasmid containing the GS-selection unit, carrying the light chain fusion gene expression casette followed by a

15   comparable heavy chain fusion gene expression casette. A schematic representation of both plasmids is given in figures 17 and 18. The approach of assembling a single expression plasmid containing separate transcription units for both heavy and light chains and the selectable marker, is adviced in order to ensure coamplification with the marker gene. The cDNA sequence encoding the complete MoTAbII heavy chain fusion protein is given in figure 19. The amino

20   acid sequence of the MoTAbII heavy chain fusion protein is given in figure 20.

Small scale expression of D9D10 MoTAbII in COS cells

Transient expression in COS monkey kidney cells was performed using both mammalian

25   expression constructs pEE12MoTAbII and pEE14MoTAbII completely as decribed for the humanized D9D10 antibody (cf example 2). Quality control was performed by IFNγ-binding ELISA and SPR-analysis.

ELISA

30   The same set up was used as described in example 2. Results are shown in figure 11. Specific binding to IFNγ is detected. The signal is lower than the signal obtained with crude

COS supernatant of humanized D9D10. However, no concentrations were determined of MoTAbII.

SPR analysis

5   A similar set up was used as described for the evaluation of the murine and humanized scFvD9D10 derivatives. Briefly, murine D9D10 was immobilized directly onto a B1 sensorchip at a concentration of 10 µg/ml D9D10 in an acetate buffer pH 4.8 using amine coupling. A fixed concentration of 8 µg/ml human IFNγ was added, followed by the injection of either murine D9D10 (10 µg/ml; positive control) or crude COS supernatant containing MoTAb II. Results

10   are shown in figure 21. These data clearly illustrate the presence of active, IFNγ binding molecules in the COS supernatant. As no exact concentrations were determined of the MoTAB II, no affinity data could be calculated.

Inhibition of MHC class II induction

15   cf example 8.1.

* *Purification*

MoTAbII was purified using classical protein A chromatography (Perry and Kirby, 1990;

20   Page and Thorpe, 1996). Quality control of the purified construct was done by Western Blot (classical technology) and ELISA. The latter was performed as described in example 2 and results are shown in figure 13. From these results we can conclude that MoTAbII is specifically interacting with human IFNγ.

25   * *Generation of stable mammalian expression cell lines*

For generation of stable mammalian expression cell line, two host cell lines Ns0 (Galfre and Milstein, 1981; ECACC 85110503) and CHO-K1 (ATCC CCL61) were used. Transfection and selection procedures were completely identical as described for the humanized D9D10

30   whole antibody, using the plasmids pEE12MoTAbII for Ns0 and pEE14MoTAbII for CHO-K1. For both NS0 and CHO-K1, several MoTAbII producing cell lines (determined in IFNγ-binding

ELISA) were initially isolated and used as parental clones for further amplification of recombinant protein expression levels as described earlier.

Production of large amounts of the recombinant protein is performed on bioreactor systems optimal for the respective host cells.

5

5. Generation of anti-IFNγ diabodies

Diabodies are dimeric antibody fragments. In each polypeptide, a heavy-chain variable domain (V_H) is linked to a light-chain variable domain (V_L) but unlike scFv's, each antigen-

10   binding site is formed by pairing of one V_H and one V_L domain from two different polypeptides. This is achieved by shortening the linker between the V_H and V_L domains in each molecule (Holliger et al., 1993). Since diabodies have two antigen-binding sites they can either be monospecific or bispecific. Monospecific bivalent molecules are generated by the shortening the flexible linker sequence of the scFv molecule to between five and ten residues

15   and by cross-pairing 2 scFv molecules with shortened linker. In order to stabilise the molecule, an optional cysteïne residue can be inserted in the linker. As an example for the different steps involved in such a construction we have documented the construction of D9D10-derived monospecific, humanized anti-IFNγ diabodies. The 15 residue linker of the His6-tagged, humanized scFvD9D10 was replaced by the 5 or 10 residue linker using overlap extension PCR.

20   Shortly, both D9D10 V_H and V_L coding sequences were PCR amplified whereby the V_H antisense primer and the V_L sense primer have sequences coding for the 5- or 10-mer linker sequence. The resulting V_H and V_L PCR fragments were subsequently mixed and a second PCR with the V_H sense and V_L antisense primers was performed. The resulting PCR fragment is cloned into the pBSK(+) plasmid (Stratagene) en verified by DNA sequence analysis (figure 22-

25   25). The D9D10 diabody coding sequence was subsequently transferred as a SapI blunt/EcoRI fragment and inserted into the NcoI blunt/EcoRI opened vector pTrc99A (Amann et al., 1988). In this vector, expression of the diabodies is under control of the IPTG inducible Trc promotor. The diabodies were expressed in *E. coli* strains HB101 or JM83. Periplasmic fractions were prepared following a modified protocol described by Neu and Heppel (1965). Briefly, cells were

30   harvested by centrifugation and resuspended in ice cold shockbuffer (100mM Tris-HCl pH 7.4; 20% sucrose; 1mM EDTA pH8). After incubation on ice during 10 min. with occasional stirring,

the mixture was centrifuged at 10.000rpm during 1,5 min. The supernatans was removed and the

pellet was immediately resuspended in ice cold distilled water. After incubation on ice during

10 min. with occasional stirring, the mixture was centrifuged at 14.000rpm and the obtained

supernatans was the soluble periplasmic fraction. The periplasmic fractions were tested for

5   binding to IFNγ using SPR-analysis. The experimental set up was as described in example 2.

The undiluted samples were injected onto the surface of a B1 sensorchip coated with murine

D9D10 onto which IFNγ was injected. Results obtained with L5 D9D10 diabodies are shown

in figure 26. A clear, specific binding of the diabodies was detected. Comparable results were

obtained with the L10 D9D10 diabody.

10   The bivalent, monospecific diabody molecules are purified from the periplasmic extract

via IMAC or from periplasmic inclusion bodies using denaturing conditions followed by

refolding.

Overlap extension PCR primers for the L10 D9D10 diabodies:

15   *D9D10V$_H$ forward (sense) primer*

5'-GGCCGCTCTTCGAAATACCTATTGCCTACGGCAG-3'

D9D10L10V$_H$ backward (antisense) primer

5'-CTGGGTCAGTACGATGTCAGAGCCACCTCCGCCTGAACCGCCTCCACCTG

20   AGGAGACGGTGACCGTGGTC-3'

D9D10L10V$_L$ forward (sense) primer

5'-GTCACCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGACA

TCGTACTGACCCAGAGCC-3'

25

D9D10V$_L$ backward (antisense) primer

5'-GCCAGTGAATTCTATTAGTGGTGATG-3'

Overlap extension PCR primers for the L5 D9D10 diabodies:

30   .   *D9D10V$_H$ forward (sense) primer*

5'-GGCCGCTCTTCGAAATACCTATTGCCTACGGCAG-3'

D9D10L5V_H backward (antisense) primer

5'-CTGGGTCAGTACGATGTCTGAACCGCCTCCACCTGAGGAGACGGTGACCGT
GGTC-3'

5   *D9D10L5V_L forward (sense) primer*

5'-GTCACCGTCTCCTCAGGTGGAGGCGGTTCAGACATCGTACTGACCCAGAGCC-3'

           D9D10V_L backward (antisense) primer

5'-GCCAGTGAATTCTATTAGTGGTGATG-3'

10

6. Generation of anti-IFNγ triabodies

           The construction of triabody molecules was analogous to the scheme described above for

           diabody molecules, except that the $(G_4S)_3$ linker between the humanized D9D10 VH and VL was

15   completely deleted (figures 27 and 28)(zero-residue linker or -1-residue linker according to the

           Kabat numbering (Kortt et al., 1997; Iliades et al., 1997)). The humanized D9D10 triabody

           construct is a mono-specific molecule resulting from the spontaneous association of three zero-

           residue linker (or -1-residue) D9D10 scFv molecules in the bacterial periplasm. A trimer was

           formed whereby three pairs of V_H and V_L domains interact to form three active antigen

20   combining sites. If necessary, in order to drive triabody formation as well as to maintain

           stability, we can explore the possibility of introducing additional association domains or

           disulfide bridges.

           The produced triabodies were tested for IFNγ binding using SPR-analysis. Periplasmic

           fractions were prepared as described in example 5. SPR-analysis was performed as described

25   in example 5. Results are shown in figure 29. A clear, specific binding of the triabody was

           obtained.

           The triabody molecules were purified from the periplasmic extract, made from uninduced

           bacterial cultures, via IMAC and further by gel filtration or alternatively by purification under

           denaturing conditions from periplasmic inclusionbodies followed by refolding. The multimeric

30   behaviour of the purified molecules was analysed. The ability of the purified triabody to bind

           human interferon γ was tested using SPR-analysis and ELISA experiments as described earlier.

For these tests we produced milligram amounts of highly purified material in a suitable *E.coli* expression system.

Overlap extension PCR primers for the L0 D9D10 triabodies :

5   *D9D10V$_H$ forward (sense) primer*

5'-GGCCGCTCTTCGAAATACCTATTGCCTACGGCAG-3'

D9D10L0V$_H$ backward (antisense) primer

5'-CTGGGTCAGTACGATGTCTGAGGAGACGGTGACCGTGGTC-3'

10

D9D10L0V$_L$ forward (sense) primer

5'-GTCACCGTCTCCTCAGACATCGTACTGACCCAGAGCC-3'

D9D10V$_L$ backward (antisense) primer

15   5'-GCCAGTGAATTCTATTAGTGGTGATG-3'

7. Generation of MoTAb's (and BiTAb's) originating from fusion proteins, from serum multisubunit proteins and from scFv's

20   The multi subunit (oligomeric) structure of proteins may be exploited to obtain multivalent antibodies, when they are used as fusion partner with scFv antibodies. Either the whole polypetide chain, or the association sequence domain may be used as fusion partner.

For example, haemoglobin is a tetrameric serum protein, consisting from 2 alpha and 2 beta globin subunits. The dimer dissociation constant is estimated to be in the order of 1nM (Pin

25   et al., 1990). The tetramer - dimer dissociation constant of haemoglobin in oxy-conformation was studied by gel filtration on Superose 12 and was calculated to be 1μM (Manning et al, 1996). Although non-covalent associations are known to be susceptible to equilibrium rules, it has been described that the subunit interactions are favoured in concentrated protein solutions like serum and also may be increased by the presence of other stabilising compounds (Srere and

30   Mathews, 1990).

Recombinant haemoglobin expression has been extensively investigated as a possible

blood substitute in order to circumvent the transmission of infectious disease agents during blood transfusion. The alpha- and beta - globin polypeptides have already been expressed from a single operon in *E. coli* (Hoffman et al., 1990). In this case, the recombinant haemoglobin was purified from the soluble cytoplasmatic fraction and the tetrameric *E. coli* product had essentially

5   the same characteristics as the native protein. Analogous results were obtained when recombinant haemoglobin was expressed in *S. cerevisiae* (Pagnier et al., 1992; Mould et al., 1994; Sutherland-Smith et al., 1998).

Protein engineering strategies (Olson et al., 1997) and chemical modification by pegylation (Pettit and Gombotz, 1998) are investigated to enhance the stability and the

10   circulation half times *in vivo*. So fusion of relevant scFv molecules to the respective alpha and beta subunit of human haemoglobin and expression of the fusion proteins from a single operon in either *E. coli* or *S. cerevisiae* would yield a functional tetrameric monospecific (if identical scFv's are used) or bispecific (when different scFv's are used) molecules at high level.

15   **8. Evaluation of anti-IFNγ neutralizing molecules**

8.1. Inhibition of MHCII-induction

In the first experiments, the effect of IFNγ on the induction of MHC class II expression

20   on human keratinocytes was examined. For this, primary human keratinocytes (passage 1) were cultured with two concentrations of human IFNγ (100 U/ml and 200 U/ml) during 24 and 48 hours. After culture, cells were collected and the expression of MHC class II antigen on the activated keratinocytes was measured by FACS-scan after staining (30 minutes at 4°C) of the cells with a PE-labelled anti-MHC-class II mAb. The results showed that resting keratinocytes

25   do not express MHC class II molecules and that IFNγ induces the expression after 24 hours in a dose-dependent way. The induction is still enhanced after 48 hours of culture.

In the next study, the effect of anti-human IFNγ D9D10H3 full size antibody or scFvD9D10-cmyc on the IFNγ-induced MHC-Class II expression on human keratinocytes was examined. In this experiment, human primary keratinocytes (passage 1) were cultured with

30   human IFNγ (100 U/ml) in the presence or absence of different concentrations (2- 0.5- 0.12- 0.03) D9D10 Ab or D9D10scFv for 48 hours. IFNγ was preincubated with D9D10H3 or

scFvD9D10 during 1 hour at 37°C before adding to the keratinocytes. After culture, cells were collected and the expression of MHC-Class II on these activated keratinocytes was measured. For this, keratinocytes were incubated (30 minutes at 4°C) with a PE-labelled anti-MHC-ClassII mAb (Becton Dickinson), washed twice with PBS and fixed. The MHC-Class II expression was further analysed on a FACS-scan. The results of these experiments are represented in figure 30. It is shown that the MHC class II antigen is not expressed on the membrane of resting keratinocytes and that IFNγ clearly induces this MHC class II expression. This IFNγ induced MHC class II expression is dose dependently inhibited by D9D10H3 and to a lesser extent by scFvD9D10. We can conclude that about 4 times more scFv (0.12 µg/ml) than full size antibody (0.5 µg/ml) is needed to obtain a 50 % inhibition of the IFNγ-induced MHC classII expression on keratinocytes.

Similar experiments were performed in order to evaluate the neutralization capacity of humanized D9D10 and MoTAbII. Results are summarized in figure 31. Although in this experiment, MHC class II induction could be only induced to a lesser extent, both humanized D9D10 and MoTAbII clearly inhibit the IFNγ-induction.

8.2. Inhibition of anti-viral activity

For neutralization of the antiviral activity of hIFNγ, serial dilutions of samples (anti-IFNγ constructs) were prepared in microtiter plates. To each well, hIFNγ was added to a final concentration of 5 antiviral protection Units/ml, as tested on A549 cells. The mixtures were incubated for 4 h at 37°C and 25000 A549 cells were added to each well. After an incubation period of 24 at 37°C in a CO_2 incubator, 25 µl of 8×10^5 PFU EMC virus/ml was added to the cultures for at least 24h. As soon as virus-infected control cultures reached 100% cell destruction, a crystal violet staining was performed in order to quantify surviving cells. The neutralization capacity of the anti-IFNγ constructs was defined by the concentration of the construct needed to neutralize 95% of the antiviral activity of 5U/ml human IFNγ. The neutralization potency of the scFvD9D10 and the humanized scFvD9D10 was determined and was 1.2 µg/ml and 1.5 µg/ml, respectively.

8.3. Beneficial effects in septic shock in mice

Septic shock has been demonstrated to be a complex human disease manifestation that occurs after the release of lipopolysaccharide (LPS) into the circulation. The subsequent

5   production of high cytokine levels in the serum are known to play a crucial role in septic shock. We generated data in a mouse model system using an anti-mouse IFNγ called F3 (Froyen et al., 1995).

The generalized Shwartzman model is a lethal shock syndrome in experimental animals which is elicited by 2 consecutive injections of LPS. In the laboratory of prof. Billiau (Rega

10   Institute, Catholic University Leuven, Belgium), such a model was developed in mice (Billiau et al., 1987). At time 0, the mice were injected with 5 μg LPS into the footpad, followed 24 h later by a second intravenous injection of 100 μg. Morbidity and mortality was scored for 5 days. Untreated animals normally died within 2 days after the second injection. Mice pretreated with the anti-muIFNγ antibody F3 were completely protected against the lethal effect and only

15   showed moderate disease symptoms. This protection could be achieved with as little as 2.4 μg F3 given 24h before the first injection. In order to score the severity of the disease, the symptoms were classified in 5 groups :

Score 0 : not sick or mild piloerection

Score 1 : piloerection and diarrhoea

20   Score 2 : hemorhagic conjunctivitis and bleeding at the mouth and anus

Score 3 : paralysis of the hind legs

Score 4 : death

The highest score that could be obtained is 4. Since the number of mice in each group was relatively low (5), we established a limit of the disease score (=2) that had to be reached in the

25   saline group in order to be a representative experiment.

The schedule we used in order to compare F3 and its scFv in this Shwartzman model was as follows: NMRI mice were given the preparative dose of 5 μg LPS at time 0. At the time points +6h, +12h and +23h the mice were injected ip with 190μg scFvF3 (Froyen et al., 1995) or 30 μg F3. Control animals were given saline at the same time points. Each group consisted of 5 mice.

30   The mice were given a score according to the above mentioned classification.

In the first experiment, 40% more mice were protected in the scFvF3 group when

68

compared with the control group. A second experiment was set up using a slightly adapted protocol: an additional injection was given at timepoint +3h. The result of this experiment (shown in table) was similar to that of experiment 1 in that 40% more mice survived in the scFvF3 group in comparison with the control group as can be seen in figure 32. In addition to scFvF3, a Fab antibody fragment of F3 was included in the second group. All these mice survived the experiment.

The mean disease scores of these experiments, demonstrate a significant difference for both F3 and the scFv compared to the control group.

The mean disease scores of the 5 mice of each group were as follows:

	Saline	scFvF3	F3	FabF3
exp. 1	3.2	1.8	0.0	ND
exp. 2	2.6	0.8	0.6	0.6

8.4. Beneficial effects during cachexia in mice

In a model for cachexia developed at the Rega Institute (Matthys et al., 1991), nude mice were injected intraperitoneally (ip) with CHO cells producing mouse IFNγ (Mick cells). Mice receiving CHO-Mick cells will exhibit cachexia (including body weight loss) within 48 hours. The cachectic effect is correlated with the number of Mick cells. Thus with small tumor cell inocula ($0.8-3.0 \times 10^7$ cells), cachexia is transient and mice will completely recover. However, with high inocula ($>3.4 \times 10^7$ cells), mice continue to loose weight and will die within 7 days. It is shown that IFNγ plays an essential role in the pathogenesis of the Mick-induced cachexia as monoclonals against IFNγ can reverse the wasting effect: pretreatment (day -1) with the anti-muIFNγ antibody F3 inhibits cachexia.

In order to compare the effects of F3 and its scFv on the established cachexia model, the following experiment has been set up: mice were injected with $2-4 \times 10^7$ Mick cells on day 0 and antibody preparations were administered ip at time points +1.5h, +6h, +22h and +66h relative

to the time of Mick cell inoculation. For scFvF3, a dose of 190µg was given each injection while for F3, 40 µg was given. Control animals were injected with saline at the same time points. In each group, 3 or 4 mice were used. Mice were weighed for 10 consecutive days and mortality was scored. The results of 2 independent experiments are shown in figure 33. The mice treated

5   with scFvF3 were better protected against the cachectic effect than the control mice.

These results also indicate that scFvF3 antibody fragments do have a protective effect of cachexia but to a lesser extent than the parental F3 antibody. Although results were promising, it was clear that the effect of the scFv fragment was limited either due to its fast clearance or to lowered affinity. Optimization of the injection schedule was needed to obtain comparable results.

10

8.5. Beneficial effects in septic shock in non-human primates

The best documented sepsis model in non-human primates is the one in which baboons are given lethal infusions of *E.coli*. As described by Creasey et al. (1991), response to lethal

15   *E.coli* challenge occurs in 3 stages: an inflammatory stage marked by a fall in white blood cell count (0-2 hr) and the appearance in plasma of TNFα, IL-1β and IL-6; a coagulant stage marked by a fall in fibrinogen concentration (2-6 hr); and a hypoxic cell injury stage marked by a rise in SGPT/BUN and by a gradual cardiovascular collapse, and death (6-24 hr).

Since the baboon animal model was not readily available, we are establishing a

20   comparable rhesus monkey model. D9D10 and derived constructs interacted well with rhesus IFNγ as determined in an antiviral bioassay (set up as described in example 7.2.)

Septic shock can be induced by infusion either of life bacteria or of endotoxin in sedated monkeys. After administration of different concentrations of the D9D10 anti-hIFNγ derivatives, several parameters are monitored including :

25   - mortality (should be 100% in control (non-treated) group)

             - pathophysiology

             - serum concentration of cytokines such as TNFα, IL-1 and IL-6 using ELISA or
               bioassay (Villinger et al., 1993)

             - endotoxin profile using the limulus amoebocyte lysate assay

30

8.6. Beneficial effects during experimental autoimmune encephalomyelitis in non-human primates

A.   Pharmacokinetics of D9D10 and derivatives in monkey and effect on hIFNγ

5   **clearance**

The clearance of the antibody derivatives is of importance as molecules with a slow clearance have a prolonged efficacy. This implicates that less material has to be injected which is better for the patient and which is cost effective, especially when a longer treatment period is

10   advisable. Therefore, complexes of IFNγ and D9D10 derivatives are used in clearance studies in non-human primates as a prerequisite to guide further *in vivo* studies in these animals.

The clearance of D9D10, scFvD9D10H6⁻, D9D10 MOTAB I and D9D10 MOTAB II , is monitored after a bolus injection in healthy marmoset. Specific ELISA's are used for monitoring; no labelling of the antibody constructs is required.

15   Blood clearance of radiolabelled marmoset IFNγ after a bolus intravenous injection alone or in combination with one of the antibody constructs are also performed.

B.   Beneficial effects of the D9D10 antibody constructs on EAE in non-human primates

20   In order to evaluate the therapeutic potential of the anti-IFNγ Mab D9D10 and derivatives, we are testing this antibody in a relevant non-human primate model for MS as the final step in our preclinical research. This model is required since the antibody is not cross-reacting with IFNγ from rodents and the biological activity of IFNγ is very species specific (huIFNγ is not active on cells other than human or non-human primates (Terrell and Green,

25   1993)). D9D10 and derived constructs interact well with marmoset IFNγ as determined in an antiviral bioassay (set up as described in example 7.2.) and using surface plasmon resonance (set up as described in example 1).

The EAE model is chosen as it is a generally accepted model for Multiple Sclerosis. We opt for the EAE model in common marmoset (*Callithrix jacchus*) as it is well developed

30   (Massacesi et al., 1995; Genain et al., 1995), it has a pathology of MR-detectable lesions which reflects those in MS and the model shows a high incidence of EAE induction with a chronic

progressive/relapsing-remitting course.

<u>Acute PK-Tox</u>

5   A limited PK-Tox study required by ethical prescriptions in all research involving non-human primates, is set up in order to test the toxicity of the substances administered either intravenously or in the lumbar cerebrospinal fluid (CSF), as the contribution of systemic and/or local IFNγ to the development of the disease is still unclear. Relatively high concentrations of the antibody preparations, especially for the scFv, are injected intravenously as one of our goals

10   is to reach therapeutical concentrations in the CNS. Although it is known that BBB leakage occurs at the site of inflammation ('t Hart, personal communication), a positive concentration gradient will be beneficial.

<u>Timing of the study</u>

15

        Determination of the baseline parameters is done 1 week prior to administration of the study drug. Animals are observed for signs of toxicity for 30 days. During this period pharmacokinetic parameters are monitored. Six weeks after the administration of the study drug an additional blood sample is collected to determine whether or not the animals mounted an

20   immune response to any of the D9D10 constructs or to recombinant marmoset IFNγ.

<u>Parameters</u>

        During this study the following parameters are determined:

25   *Clinical monitoring*

            Daily   : Food consumption
            Weekly   : Body weights
            Day 14, 28   : Haematology
                            Clinical chemistry
30   Urine analysis

Immunological monitoring

Serum and CSF levels of humanized D9D10, MoTAbI and II or IFNγ are measured at different time points. When severe toxicity occurs in one of the animals, the animal are sacrificed and subjected to a detailed necropsy, in order to determine whether this toxicity is drug related.

5

Diffusion of D9D10 derivatives into the lesions

As both systemically and locally (in the brain) produced IFNγ can have a disease promoting role in EAE, antibody derivatives must be able to neutralize both. Consequently,

10   transudation of the D9D10 derivatives into the lesions in brain and spinal cord is necessary for a local effect on IFNγ. However, it is known that in MS the blood brain barrier is impaired in a subset of the active brain lesions for a limited period of time. More specifically, BBB breakdown is reflecting the state of inflammation (Hawkins et al., 1990).

The differential ability of the anti-IFN constructs to enter the brain is crucial for the

15   choice of the component(s) which will be used for evaluation of the therapeutic efficacy of an anti-IFNγ treatment in EAE.

The entry of the constructs into the brain compartment is measured by post-mortem magnetic resonance imaging (MRI)-scan of the brain and the spinal cord of a relapsing monkey, injected intravenously with a gadolinium-diethylene-triamine pentaacetic acid (Gd-DTPA)-

20   labelled D9D10 construct 1 hour prior to sacrifice. MRI-scans are compared and are related to an MRI-scan taken just before death after an injection with a small gadolinium salt that easily enters through leakages in the BBB (Gonzalez-Scarano et al., 1987; Hawkins et al., 1990; Youl et al, 1991).

These results reveal which D9D10 construct most easily enters the brain and which

25   molecule eventually enters the active lesions where the BBB is already restituted.

Therapeutic treatment of marmoset monkeys undergoing EAE disease relapse

The therapeutic effect of either systemically or locally administered anti-IFNγ on the

30   outcome of EAE in marmoset is evaluated. The start of the treatment of the monkeys is situated at the beginning of the first relapse of EAE, which usually occurs several months after the initial

immunization. During the experiment the following observations, analysis and measurements are carried out as of the time of relapse.

Clinical monitoring

5   - The severity of EAE is scored daily on an arbitrary scale modified from Massacesi et

al. (1995)

- Body weight and body temperature (at time of blood sampling)

- Behavioural tests for monitoring the failure of neurological functions

- Magnetic resonance imaging (MRI) of the CNS

10   - Biochemical parameters: neopterin (specifically formed in activated macrophages) is

measured in urine

Immunological monitoring

At several indicated time points serum is taken to monitor the blood levels of the

15   antibody constructs or IFNγ and to monitor the marmoset anti-mouse or anti-IFN response.

Pathology

MRI-guided histopathology analysis has proven a powerful tool for detailed analysis of

MR-detectable lesions with histological methods. Briefly, at a chosen moment but preferably

20   shortly after *in vivo* MR-images have been recorded, the monkey are euthanised. The brain and

spinal cord is carefully excised and fixed *in toto* for 3 days in 4% buffered formaldehyde. Then

a T2-weighted scan is made in axial and coronal direction, with a slice thickness of 1 mm

covering the whole brain. For orientation of the axial slices of *in vivo* and *in vitro* images the

anterior and posterior tips of the corpus callosum are used as internal reference points.

25   The excellent structural conservation and the high resolution of the MR-image make

accurate three-dimensional localisation of potential lesions possible. Regions of interest are

subsequently excised and histologically analysed for infiltrating cells (Haematoxylin-eosin),

demyelisation (KLB staining of myelin lipids) and axonal structure (silver impregnation acc. to

Boielschowsky).

30   One half of an excised brain and spinal cord is snap-frozen in liquid nitrogen. Thin

cryosections are made and processed for immunohistology staining, such as for visualisation of

cytokine secreting cells (especially IFNγ) or for phenotyping of infiltrated or tissue cells.

8.7. Beneficial effects of anti-IFNγ antibody constructs in Crohn's disease

5   A.   *In vitro* **assay using patient-derived lymphocytes and antigen presenting cells**

Lymphocytes isolated from either peripheral blood or surgical specimen (lamina propria or ileum E) from patients with Crohn's disease, are used for assessment of cytokine profile, lymphotyping, and functional cytotoxicity. The latter is performed by adding patient-derived

10   antigen presenting cells and measuring the cytokine profile. The effect of anti-IFNγ derived antibody constructs on cytokine production is measured.

B   Anti-IFNγ treatment of Crohn's disease

15   Patients with active Crohn's disease are infused with anti-IFNγ in a dose ranging from 1 to 20 mg/kg. Responders in the study may continue to receive repeated doses of anti-IFNγ. In all patients, clinical responses are observed and Crohn's disease activity index (CDAI) is determined.

20

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CLAIMS

1. A molecule which binds and neutralizes interferon-gamma and which is chosen from the group consisting of:

- a scFv comprising the humanized variable domain of the monoclonal antibody D9D10

- a chimeric antibody comprising the humanized variable domain of the monoclonal antibody D9D10

- a diabody comprising the humanized variable domain of the monoclonal antibody D9D10

- a multivalent antibody

- a ruminant antibody.

2. A molecule according to claim 1, wherein said ruminant antibody is a sheep antibody.

3. A molecule according to claim 2, wherein said sheep antibody is a monoclonal antibody.

4. A molecule according to claim 3, wherein said monoclonal antibody is a humanized antibody, a single-chain fragment or any other fragment thereof which has largely retained the specificity of said antibody.

5. A molecule according to claim 1, wherein said multivalent antibody is chosen from the group consisting of triabodies, tetravalent antibodies, peptabodies and hexabodies.

6. A molecule according to claim 5, wherein said triabody, tetravalent antibody, peptabody and hexabody comprise 3, 4, 5 and 6 variable domains, respectively, of several anti-interferon-gamma antibodies.

7. A molecule according to claim 5, wherein said triabody comprises 3 identical variable domains of an anti-interferon-gamma antibody.

8. A molecule according to claim 5, wherein said triabody comprises 3 identical D9D10 scFv's or 3 identical sheep-derived anti-interferon-gamma scFv's.

9. A molecule according to claim 5, wherein said triabody comprises 3 identical humanized D9D10 scFv's or 3 identical humanized sheep-derived anti-interferon-gamma scFv's.

10. A molecule according to claim 5, wherein said tetravalent antibody comprises 4 identical domains of an anti-interferon-gamma antibody.

11. A molecule according to claim 5, wherein said tetravalent antibody comprises 4 identical D9D10 scFv's or sheep-derived anti-interferon-gamma scFv's in the format of a homodimer of 2 identical molecules, each containing 2 D9D10 scFv's or 2 sheep-derived anti-interferon-gamma scFv's, and a dimerization domain.

12. A molecule according to claim 5, wherein said tetravalent antibody comprises 4 identical humanized D9D10 scFv's or humanized sheep-derived anti-interferon-gamma scFv's in the format of a homodimer of 2 identical molecules, each containing 2 humanized D9D10 scFv's or 2 humanized sheep-derived anti-interferon-gamma scFv's, and a dimerization domain.

13. A molecule according to claim 5, wherein said tetravalent antibody comprises a full-size humanized D9D10 antibody or humanized sheep-derived antibody to which 2 humanized D9D10 scFv's or 2 humanized sheep-derived anti-interferon-gamma scFv's are attached, respectively, at the carboxyterminus.

14. A molecule according to claim 5, wherein said peptabody and hexabody comprise 5 and 6 identical variable domains of an anti-interferon-gamma antibody, respectively.

15. A molecule according to claim 5, wherein said peptabody and hexabody comprise 5 and 6 identical D9D10 scFv's or 5 and 6 identical sheep-derived anti-interferon-gamma scFv's, respectively.

16. A molecule according to claim 5, wherein said peptabody and hexabody comprise 5 and 6 identical humanized D9D10 scFv's or 5 and 6 identical humanized sheep-derived anti-interferon-gamma scFv's, respectively.

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17. A method for producing a molecule according to claims 1-16.

18. A pharmaceutical composition comprising a molecule according to any of claims 1-16 or a mixture of said molecules in a pharmaceutically acceptable excipient.

5

19. A molecule according to any of claims 1 to 16 or a composition according to claim 18 for use as a medicament.

20. A molecule according to claims 1 to 16 or a composition according to claim 18 for
10 preventing or treating septic shock, cachexia, immune diseases such as multiple sclerosis and Crohn's disease and skin disorders such as bullous, inflammatory and neoplastic dermatoses.

21. A molecule according to claims 1 to 16 for determining interferon gamma levels in a sample.

Figure 1

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Figure 2

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ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
MET Lys Tyr Leu Leu Pro Thr Ala Ala Ala Gly Leu Leu Leu Leu


GCT GCC CAA CCA GCG ATG GCC CAG GTG CAG CTG GTG CAG AGC GGT
Ala Ala Gln Pro Ala MET Ala Gln Val Gln Leu Val Gln Ser Gly


AGC GAA CTG AAA AAA CCG GGT GCG AGC GTT AAG ATC AGC TGC AAA
Ser Glu Leu Lys Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys
                                                   CDR1
GCG AGC GGT TAT ACC TTC ACC GAT TAC GGT ATG AAC TGG GTT AAA
Ala Ser Gly Tyr Thr Phe Thr Asp Tyr Gly MET Asn Trp Val Lys


CAG GCG CCG GGT CAA GGT CTG AAA TGG ATG GGT TGG ATC AAC ACC
Gln Ala Pro Gly Gln Gly Leu Lys Trp MET Gly Trp Ile Asn Thr
                  CDR2
TAC ACC GGT GAA AGC ACC TAC GTT GAC GAT TTC AAA GGT CGT TTC
Tyr Thr Gly Glu Ser Thr Tyr Val Asp Asp Phe Lys Gly Arg Phe


GTT TTC AGC CTG GAT ACC AGC GTT AGC GCG GCC TAC CTG CAG ATC
Val Phe Ser Leu Asp Thr Ser Val Ser Ala Ala Tyr Leu Gln Ile


AGC TCT CTG AAA GCG GAA GAC ACC GCG ACC TAC TTC TGC GCG CGT
Ser Ser Leu Lys Ala Glu Asp Thr Ala Thr Tyr Phe Cys Ala Arg
          CDR3
CGC GGT TTC TAC GCG ATG GAT TAC TGG GGC CAA GGG ACC ACG GTC
Arg Gly Phe Tyr Ala MET Asp Tyr Trp Gly Gln Gly Thr Thr Val
                                        Linker
ACC GTC TCC TCA GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGC
Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly


GGT GGC GGA TCG GAC ATC GTA CTG ACC CAG AGC CCG GCG ACC ATG
Gly Gly Gly Ser Asp Ile Val Leu Thr Gln Ser Pro Ala Thr MET


AGC GCG AGC CCG GGT GAA CGT GTT ACC CTG ACC TGC AGC GCG AGC
Ser Ala Ser Pro Gly Glu Arg Val Thr Leu Thr Cys Ser Ala Ser
```

Figure 2 cont'd

CDR1

TCT AGC ATC AGC TAT ATG TTC TGG TAT CAT CAG CGT CCG GGT CAG
Ser Ser Ile Ser Tyr MET Phe Trp Tyr His Gln Arg Pro Gly Gln

CDR2

AGC CCG CGT CTG TTG ATC TAT GAT ACC AGC AAC CTG GCG AGC GGT
Ser Pro Arg Leu Leu Ile Tyr Asp Thr Ser Asn Leu Ala Ser Gly

GTT CCG GCG CGT TTC AGC GGT AGC GGT AGC GGT ACC AGC TAT AGC
Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser

CTG ACC ATC AGC CGT ATG GAA CCG GAA GAT TTC GCG ACC TAT TTC
Leu Thr Ile Ser Arg MET Glu Pro Glu Asp Phe Ala Thr Tyr Phe

CDR3

TGC CAT CAG AGC TCT AGC TAT CCG TTC ACC TTC GGT CAG GGT ACC
Cys His Gln Ser Ser Ser Tyr Pro Phe Thr Phe Gly Gln Gly Thr

His6-Tag

AAA CTC GAG ATC AAA CGG CAC CAT CAC CAT CAC CAC TAA
Lys Leu Glu Ile Lys Arg His His His His His His ---

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

```
   1    ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCA
  41    GTGCCTCAGTCATACTCTCGCAGGTGCAGCTGGTGCAGAG
  81    CGGTAGCGAACTGAAAAAACCGGGTGCGAGCGTTAAGATC
 121    AGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
 161    TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATG
 201    GATGGGTTGGATCAACACCTACACCGGTGAAAGCACCTAC
 241    GTTGACGATTTCAAAGGTCGTTTCGTTTTCAGCCTGGATA
 281    CCAGCGTTAGCGCGGCCTACCTGCAGATCAGCTCTCTGAA
 321    AGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
 361    TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCA
 401    CCGTCTCGAGCGCATCCACCAAGGGCCCATCGGTCTTCCC
 441    CCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCG
 481    GCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
 521    TGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGT
 561    GCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTAC
 601    TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGG
 641    GCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAG
 681    CAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGT
 721    GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAAC
 761    TCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACC
 801    CAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACA
 841    TGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCA
 881    AGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGC
 921    CAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTAC
 961    CGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGC
1001    TGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGC
1041    CCTCCCAGCCTCCATCGAGAAAACCATCTCCAAAGCCAAA
1081    GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAT
1121    CCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTG
1161    CCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAG
1201    TGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCA
1241    CGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTA
1281    TAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGG
1321    AACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACA
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Figure 7 cont'd

1361   ACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAA
1401   GCTT

Figure 8

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  1   ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCA
 41   GTGCCTCAGTCATACTCTCGGACATCGAGCTGACCCAGAG
 81   CCCGGCGACCATGAGCGCGAGCCCGGGTGAACGTGTTACC
121   CTGACCTGCAGCGCGAGCTCTAGCATCAGCTATATGTTCT
161   GGTATCATCAGCGTCCGGGTCAGAGCCCGCGTCTGTTGAT
201   CTATGATACCAGCAACCTGGCGAGCGGTGTTCCGGCGCGT
241   TTCAGCGGTAGCGGTAGCGGTACCAGCTATAGCCTGACCA
281   TCAGCCGTATGGAACCGGAAGATTTCGCGACCTATTTCTG
321   CCATCAGAGCTCTAGCTATCCGTTCACCTTCGGTCAGGGT
361   ACCAAACTCGAGATCAAACGGACTGTGGCTGCACCATCTG
401   TCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGG
441   AACTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCC
481   AGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCC
521   AATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAG
561   CAAGGACAGCACCTACAGCCTCAGCAGCACCCTGACGCTG
601   AGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCG
641   AAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAG
681   CTTCAACAGGGGAGAGTGC
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                    5                10               15               20

                    |                |                |                |

  1    M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S  V  I  L  S
 21    Q  V  Q  L  V  Q  S  G  S  E  L  K  K  P  G  A  S  V  K  I
 41    S  C  K  A  S  G  Y  T  F  T  D  Y  G  M  N  W  V  K  Q  A
 61    P  G  Q  G  L  K  W  M  G  W  I  N  T  Y  T  G  E  S  T  Y
 81    V  D  D  F  K  G  R  F  V  F  S  L  D  T  S  V  S  A  A  Y
101    L  Q  I  S  S  L  K  A  E  D  T  A  T  Y  F  C  A  R  R  G
121    F  Y  A  M  D  Y  W  G  Q  G  T  T  V  T  V  S  S  A  S  T
141    K  G  P  S  V  F  P  L  A  P  S  S  K  S  T  S  G  G  T  A
161    A  L  G  C  L  V  K  D  Y  F  P  E  P  V  T  V  S  W  N  S
181    G  A  L  T  S  G  V  H  T  F  P  A  V  L  Q  S  S  G  L  Y
201    S  L  S  S  V  V  T  V  P  S  S  S  L  G  T  Q  T  Y  I  C
221    N  V  N  H  K  P  S  N  T  K  V  D  K  R  V  E  P  K  S  C
241    D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G  G  P  S  V
261    F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  T
281    C  V  V  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D
301    G  V  E  V  H  N  A  K  T  K  P  R  E  E  Q  Y  N  S  T  Y
321    R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G  K  E  Y  K
341    C  K  V  S  N  K  A  L  P  A  S  I  E  K  T  I  S  K  A  K
361    G  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K
381    N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E
401    W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S
421    D  G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G
441    N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S
461    L  S  L  S  P  G  K  L
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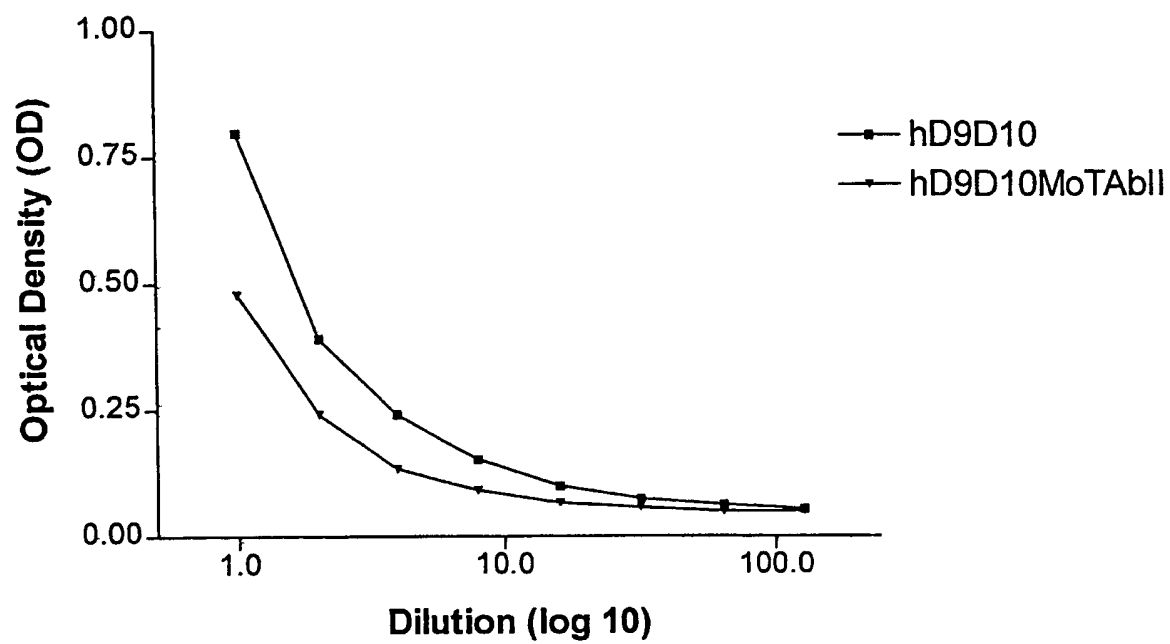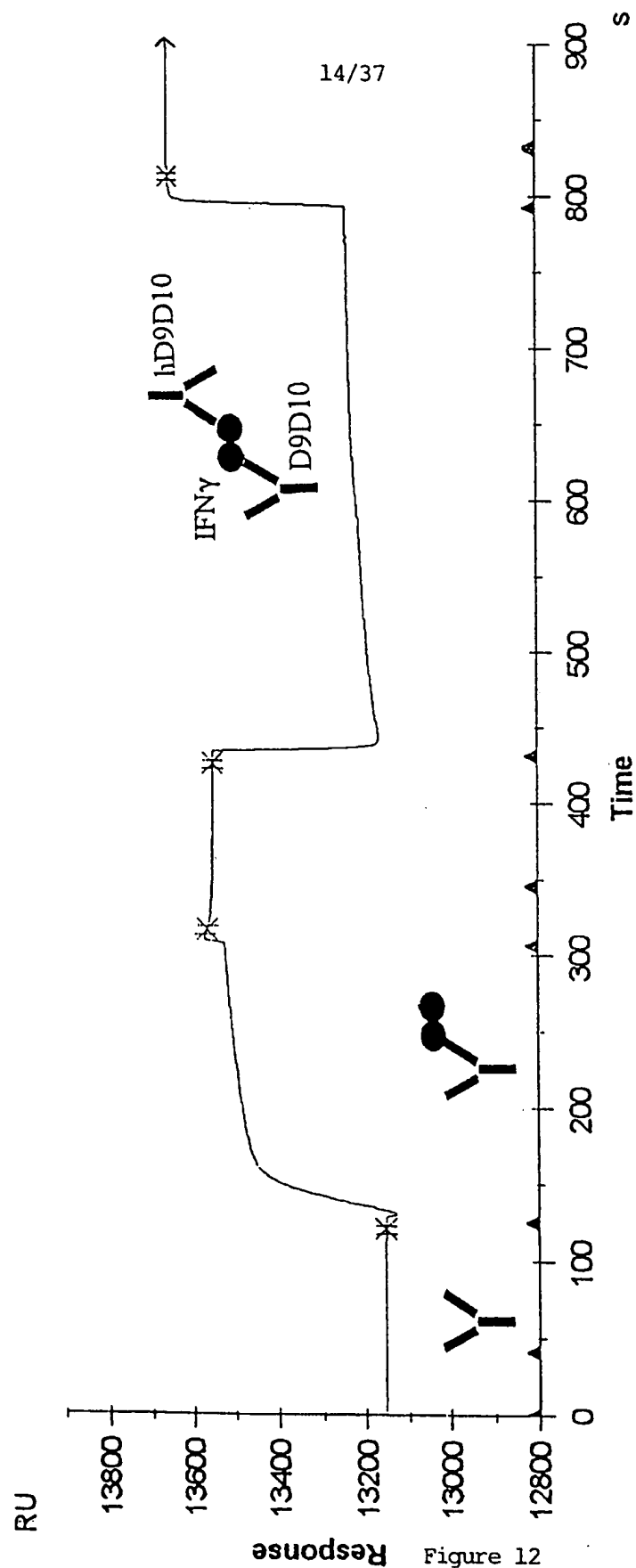
Figure 9

```
                    5              10             15             20

                    |               |              |              |

  1     M D F Q V Q I F S F L L I S A S V I L S
 21     D I E L T Q S P A T M S A S P G E R V T
 41     L T C S A S S S I S Y M F W Y H Q R P G
 61     Q S P R L L I Y D T S N L A S G V P A R
 81     F S G S G S G T S Y S L T I S R M E P E
101     D F A T Y F C H Q S S S Y P F T F G Q G
121     T K L E I K R T V A A P S V F I F P P S
141     D E Q L K S G T A S V V C L L N N F Y P
161     R E A K V Q W K V D N A L Q S G N S Q E
181     S V T E Q D S K D S T Y S L S S T L T L
201     S K A D Y E K H K V Y A C E V T H Q G L
221     S S P V T K S F N R G E C
```

Figure 10

Figure 11

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Figure 12

Figure 13

Figure 14

pMoTAb1H6
4368 bps

Figure 15

```
1    CAGGTGCAGCTGGTGCAGAGCGGTAGCGAACTGAAAAAACCGGGTGCGAG
51   CGTTAAGATCAGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
101  TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGG
151  ATCAACACCTACACCGGTGAAAGCACCTACGTTGACGATTTCAAAGGTCG
201  TTTCGTTTTCAGCCTGGATACCAGCGTTAGCGCGGCCTACCTGCAGATCA
251  GCTCTCTGAAAGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
301  TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTC
351  AGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACA
401  TCGTACTGACCCAGAGCCCGGCGACCATGAGCGCGAGCCCGGGTGAACGT
451  GTTACCCTGACCTGCAGCGCGAGCTCTAGCATCAGCTATATGTTCTGGTA
501  TCATCAGCGTCCGGGTCAGAGCCCGCGTCTGTTGATCTATGATACCAGCA
551  ACCTGGCGAGCGGTGTTCCGGCGCGTTTCAGCGGTAGCGGTAGCGGTACC
601  AGCTATAGCCTGACCATCAGCCGTATGGAACCGGAAGATTTCGCGACCTA
651  TTTCTGCCATCAGAGCTCTAGCTATCCGTTCACCTTCGGTCAGGGTACCA
701  AACTCGAGATCAAACGGACCCCGCTGGGTGATACCACTCATACCTCCGGA
751  GGTGAACTGGAAGAGCTGTTGAAACATCTGAAAGAACTGCTGAAAGGTCC
801  GCGGAAAGGTGAACTGGAGGAATTGCTGAAGCACCTGAAAGAGCTGTTGA
851  AAGGTACCCCCCTGGGTGATACTACCCATACCAGCGGTCAGGTGCAACTA
901  GTGCAGAGCGGTAGCGAACTGAAAAAACCGGGTGCGAGCGTTAAGATCAG
951  CTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTATGAACTGGGTTA
1001 AACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGGATCAACACCTAC
1051 ACCGGTGAAAGCACCTACGTTGACGATTTCAAAGGTCGTTTCGTTTTCAG
1101 CCTGGATACCAGCGTTAGCGCGGCCTACCTGCAGATCAGCTCTCTGAAAG
1151 CGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGTTTCTACGCGATG
1201 GATTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGG
1251 TTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGTACTGACCC
1301 AGAGCCCGGCGACCATGAGCGCGAGCCCGGGTGAACGTGTTACCCTGACC
1351 TGCAGCGCGAGCTCTAGCATCAGCTATATGTTCTGGTATCATCAGCGTCC
1401 GGGTCAGAGCCCGCGTCTGTTGATCTATGATACCAGCAACCTGGCGAGCG
1451 GTGTTCCGGCGCGTTTCAGCGGTAGCGGTAGCGGTACCAGCTATAGCCTG
1501 ACCATCAGCCGTATGGAACCGGAAGATTTCGCGACCTATTTCTGCCATCA
1551 GAGCTCTAGCTATCCGTTCACCTTCGGTCAGGGTACCAAACTCGAGATCA
1601 AACGGCACCATCACCATCACCACTAA
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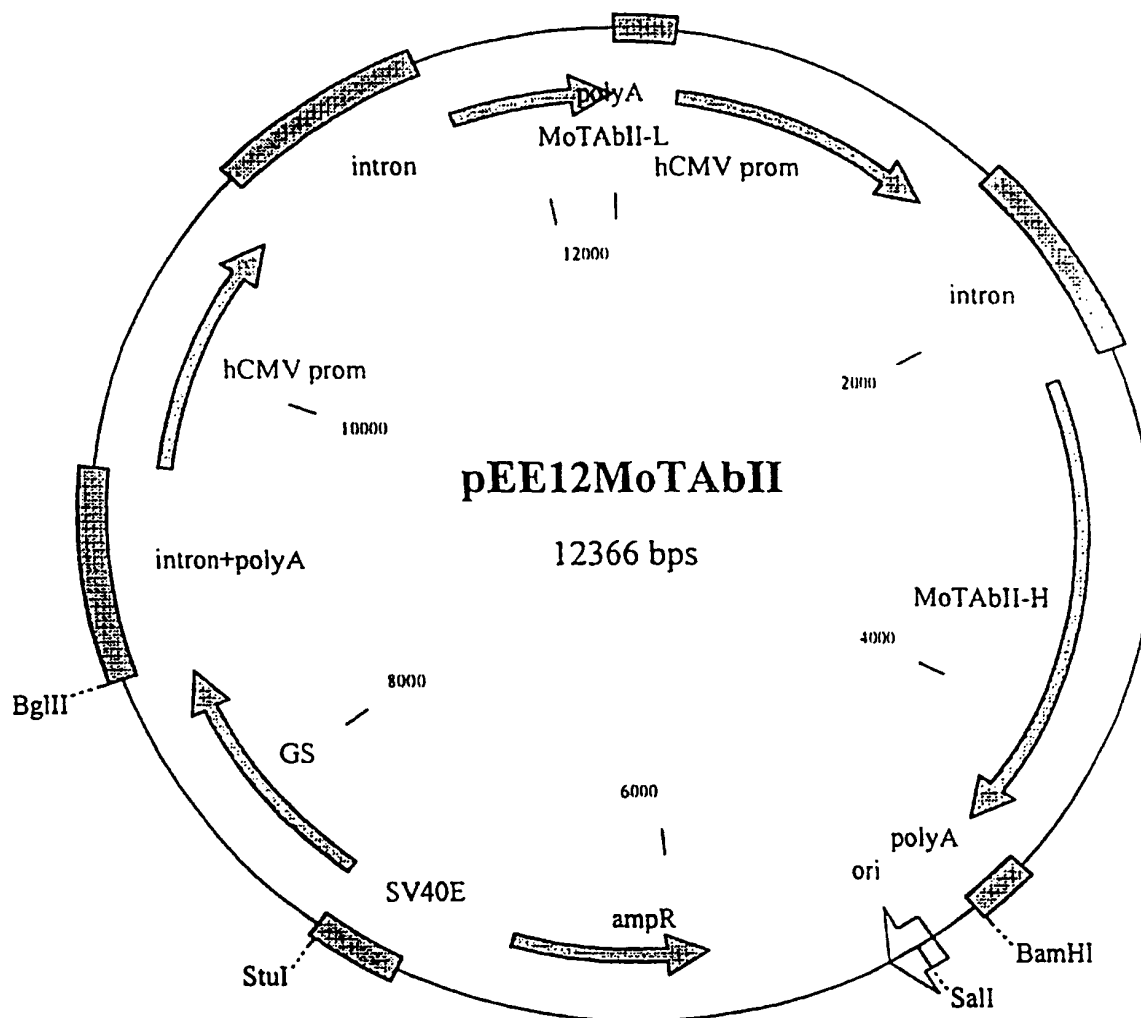
Figure 16

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51   INTYTGESTYVDDFKGRFVFSLDTSVSAAYLQISSLKAEDTATYFCARRG
101  FYAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSDIVLTQSPATMSASPGER
151  VTLTCSASSSISYMFWYHQRPGQSPRLLIYDTSNLASGVPARFSGSGSGT
201  SYSLTISRMEPEDFATYFCHQSSSYPFTFGQGTKLEIKRTPLGDTTHTSG
251  GELEELLKHLKELLKGPRKGELEELLKHLKELLKGTPLGDTTHTSGQVQL
301  VQSGSELKKPGASVKISCKASGYTFTDYGMNWVKQAPGQGLKWMGWINTY
351  TGESTYVDDFKGRFVFSLDTSVSAAYLQISSLKAEDTATYFCARRGFYAM
401  DYWGQGTTVTVSSGGGGSGGGGSGGGGSDIVLTQSPATMSASPGERVTLT
451  CSASSSISYMFWYHQRPGQSPRLLIYDTSNLASGVPARFSGSGSGTSYSL
501  TISRMEPEDFATYFCHQSSSYPFTFGQGTKLEIKRHHHHHH
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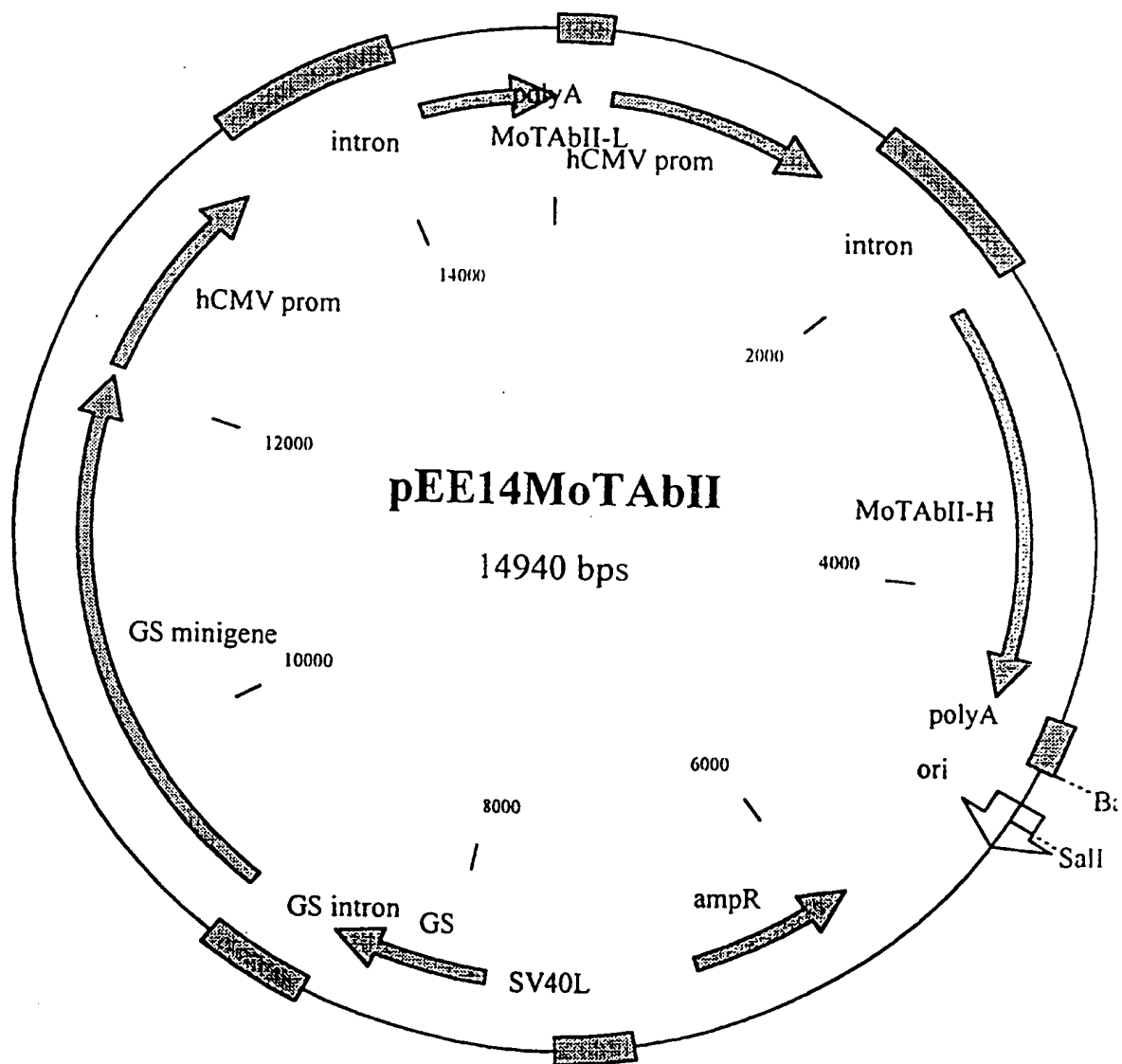
Figure 17

Figure 18

Figure 19

```
   1  ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCA
  41  GTGCCTCAGTCATACTCTCGCAGGTGCAGCTGGTGCAGAG
  81  CGGTAGCGAACTGAAAAAACCGGGTGCGAGCGTTAAGATC
 121  AGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
 161  TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATG
 201  GATGGGTTGGATCAACACCTACACCGGTGAAAGCACCTAC
 241  GTTGACGATTTCAAAGGTCGTTTCGTTTTCAGCCTGGATA
 281  CCAGCGTTAGCGCGGCCTACCTGCAGATCAGCTCTCTGAA
 321  AGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
 361  TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCA
 401  CCGTCTCGAGCGCATCCACCAAGGGCCCATCGGTCTTCCC
 441  CCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCG
 481  GCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAACCGG
 521  TGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGT
 561  GCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTAC
 601  TCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGG
 641  GCACCCAGACCTACATCTGCAACGTGAATCACAAGCCCAG
 681  CAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGT
 721  GACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAAC
 761  TCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACC
 801  CAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACA
 841  TGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCA
 881  AGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGC
 921  CAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTAC
 961  CGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGC
1001  TGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGC
1041  CCTCCCAGCCTCCATCGAGAAAACCATCTCCAAAGCCAAA
1081  GGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCAT
1121  CCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTG
1161  CCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAG
1201  TGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCA
1241  CGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTA
1281  TAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGG
1321  AACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACA
```

Figure 19 cont'd

```
1361   ACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAA
1401   GCTTGGCGGAGGCTCACAGGTGCAGCTGGTGCAGAGCGGT
1441   AGCGAACTGAAAAAACCGGGTGCGAGCGTTAAGATCAGCT
1481   GCAAAGCGAGCGGTTATACCTTCACCGATTACGGTATGAA
1521   CTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATGGATG
1561   GGTTGGATCAACACCTACACCGGTGAAAGCACCTACGTTG
1601   ACGATTTCAAAGGTCGTTTCGTTTTCAGCCTGGATACCAG
1641   CGTTAGCGCGGCCTACCTGCAGATCAGCTCTCTGAAAGCG
1681   GAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGTTTCT
1721   ACGCGATGGATTACTGGGGCCAAGGGACCACGGTCACCGT
1761   CTCCTCAGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGC
1801   GGTGGCGGATCGGACATCGTACTGACCCAGAGCCCGGCGA
1841   CCATGAGCGCGAGCCCGGGTGAACGTGTTACCCTGACCTG
1881   CAGCGCGAGCTCTAGCATCAGCTATATGTTCTGGTATCAT
1921   CAGCGTCCGGGTCAGAGCCCGCGTCTGTTGATCTATGATA
1961   CCAGCAACCTGGCGAGCGGTGTTCCGGCGCGTTTCAGCGG
2001   TAGCGGTAGCGGTACCAGCTATAGCCTGACCATCAGCCGT
2041   ATGGAACCGGAAGATTTCGCGACCTATTTCTGCCATCAGA
2081   GCTCTAGCTATCCGTTCACCTTCGGTCAGGGTACCAAACT
2121   CGAGATCAAACGG
```

Figure 20

```
                 5              10             15             20
                 |              |              |              |

  1    M D F Q V Q I F S F L L I S A S V I L S
 21    Q V Q L V Q S G S E L K K P G A S V K I
 41    S C K A S G Y T F T D Y G M N W V K Q A
 61    P G Q G L K W M G W I N T Y T G E S T Y
 81    V D D F K G R F V F S L D T S V S A A Y
101    L Q I S S L K A E D T A T Y F C A R R G
121    F Y A M D Y W G Q G T T V T V S S A S T
141    K G P S V F P L A P S S K S T S G G T A
161    A L G C L V K D Y F P E P V T V S W N S
181    G A L T S G V H T F P A V L Q S S G L Y
201    S L S S V V T V P S S S L G T Q T Y I C
221    N V N H K P S N T K V D K R V E P K S C
241    D K T H T C P P C P A P E L L G G P S V
261    F L F P P K P K D T L M I S R T P E V T
281    C V V V D V S H E D P E V K F N W Y V D
301    G V E V H N A K T K P R E E Q Y N S T Y
321    R V V S V L T V L H Q D W L N G K E Y K
341    C K V S N K A L P A S I E K T I S K A K
361    G Q P R E P Q V Y T L P P S R E E M T K
381    N Q V S L T C L V K G F Y P S D I A V E
401    W E S N G Q P E N N Y K T T P P V L D S
421    D G S F F L Y S K L T V D K S R W Q Q G
441    N V F S C S V M H E A L H N H Y T Q K S
461    L S L S P G K L G G G S Q V Q L V Q S G
481    S E L K K P G A S V K I S C K A S G Y T
501    F T D Y G M N W V K Q A P G Q G L K W M
521    G W I N T Y T G E S T Y V D D F K G R F
541    V F S L D T S V S A A Y L Q I S S L K A
561    E D T A T Y F C A R R G F Y A M D Y W G
581    Q G T T V T V S S G G G G S G G G G S G
```

Figure 20 cont'd

```
601    G G G S D I V L T Q S P A T M S A S P G
621    E R V T L T C S A S S S I S Y M F W Y H
641    Q R P G Q S P R L L I Y D T S N L A S G
661    V P A R F S G S G S G T S Y S L T I S R
681    M E P E D F A T Y F C H Q S S S Y P F T
701    F G Q G T K L E I K R
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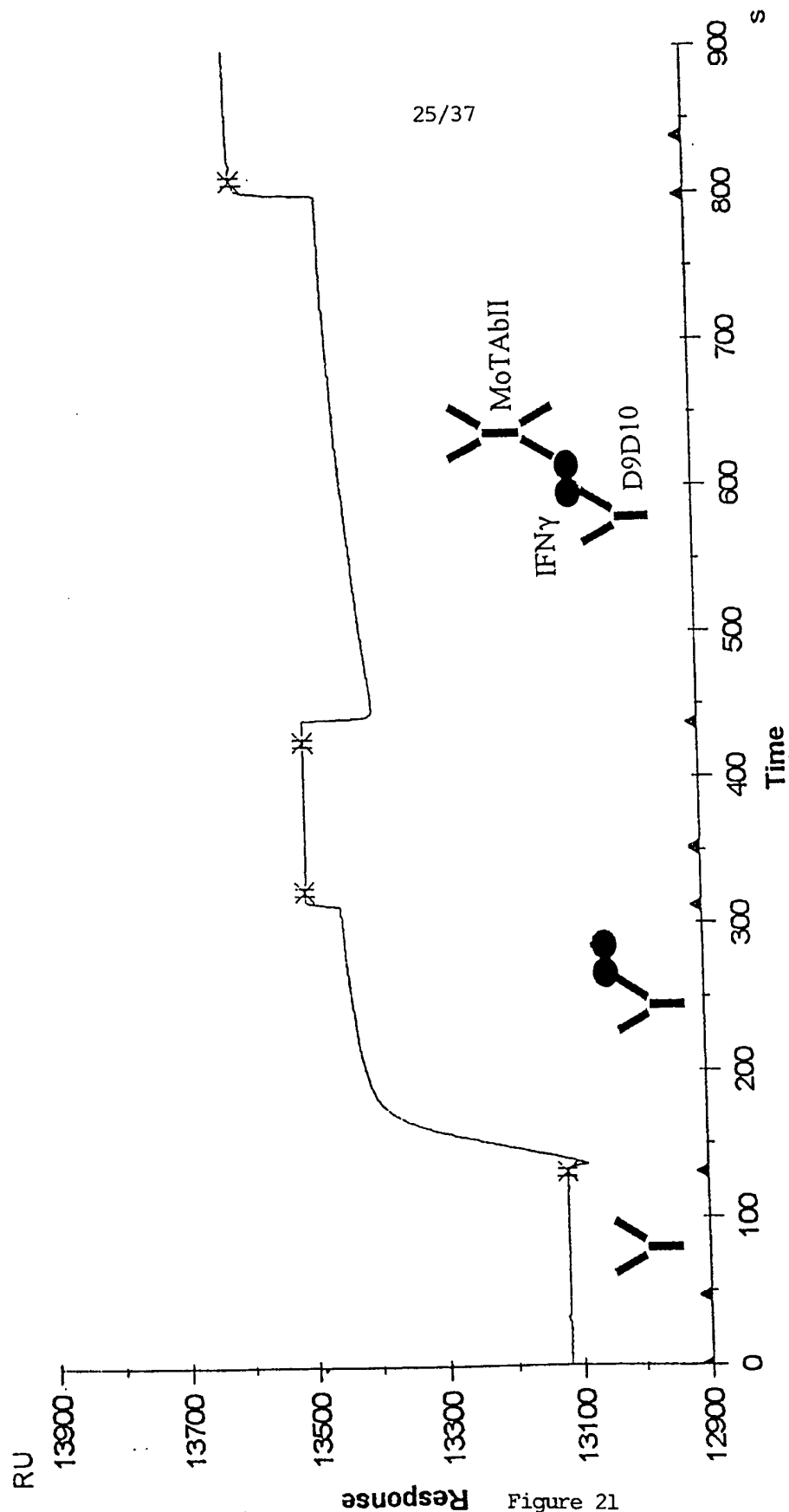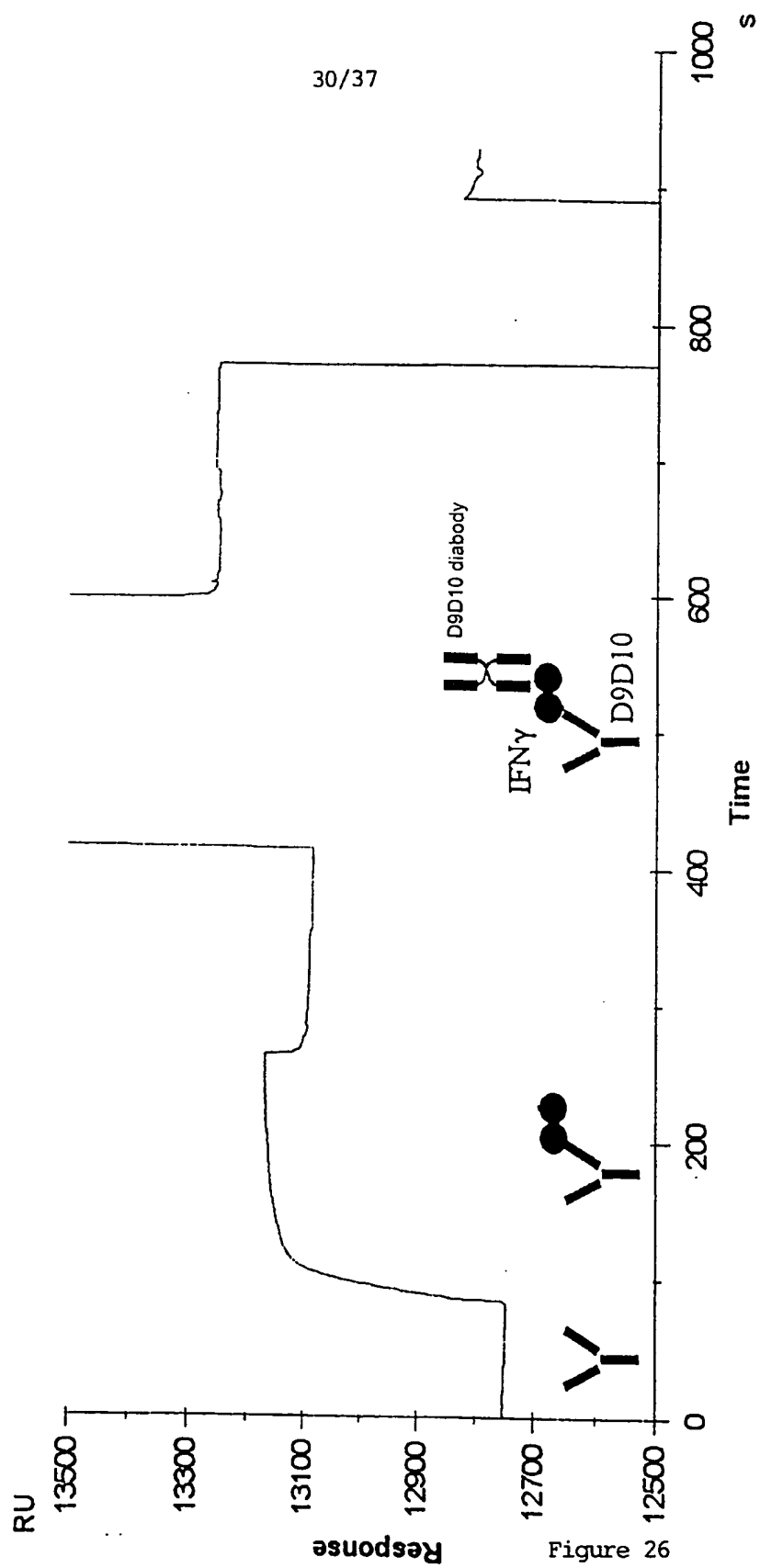
Figure 21

Figure 22

```
1    QVQLVQSGSELKKPGASVKISCKASGYTFTDYGMNWVKQAPGQGLKWMGW
51   INTYTGESTYVDDFKGRFVFSLDTSVSAAYLQISSLKAEDTATYFCARRG
101  FYAMDYWGQGTTVTVSSGGGGSGGGGSDIVLTQSPATMSASPGERVTLTC
151  SASSSISYMFWYHQRPGQSPRLLIYDTSNLASGVPARFSGSGSGTSYSLT
201  ISRMEPEDFATYFCHQSSSYPFTFGQGTKLEIKRHHHHHH
```

Figure 23

```
1     CAGGTGCAGCTGGTGCAGAGCGGTAGCGAACTGAAAAAACCGGGTGCGAG
51    CGTTAAGATCAGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
101   TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGG
151   ATCAACACCTACACCGGTGAAAGCACCTACGTTGACGATTTCAAAGGTCG
201   TTTCGTTTTCAGCCTGGATACCAGCGTTAGCGCGGCCTACCTGCAGATCA
251   GCTCTCTGAAAGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
301   TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTC
351   AGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGACATCGTACTGACCCAGA
401   GCCCGGCGACCATGAGCGCGAGCCCGGGTGAACGTGTTACCCTGACCTGC
451   AGCGCGAGCTCTAGCATCAGCTATATGTTCTGGTATCATCAGCGTCCGGG
501   TCAGAGCCCGCGTCTGTTGATCTATGATACCAGCAACCTGGCGAGCGGTG
551   TTCCGGCGCGTTTCAGCGGTAGCGGTAGCGGTACCAGCTATAGCCTGACC
601   ATCAGCCGTATGGAACCGGAAGATTTCGCGACCTATTCTGCCATCAGAG
651   CTCTAGCTATCCGTTCACCTTCGGTCAGGGTACCAAACTCGAGATCAAAC
701   GG
```

Figure 24

```
  1   QVQLVQSGSELKKPGASVKISCKASGYTFTDYGMNWVKQAPGQGLKWMGW
 51   INTYTGESTYVDDFKGRFVFSLDTSVSAAYLQISSLKAEDTATYFCARRG
101   FYAMDYWGQGTTVTVSSGGGGSDIVLTQSPATMSASPGERVTLTCSASSS
151   ISYMFWYHQRPGQSPRLLIYDTSNLASGVPARFSGSGSGTSYSLTISRME
201   PEDFATYFCHQSSSYPFTFGQGTKLEIKRHHHHHH
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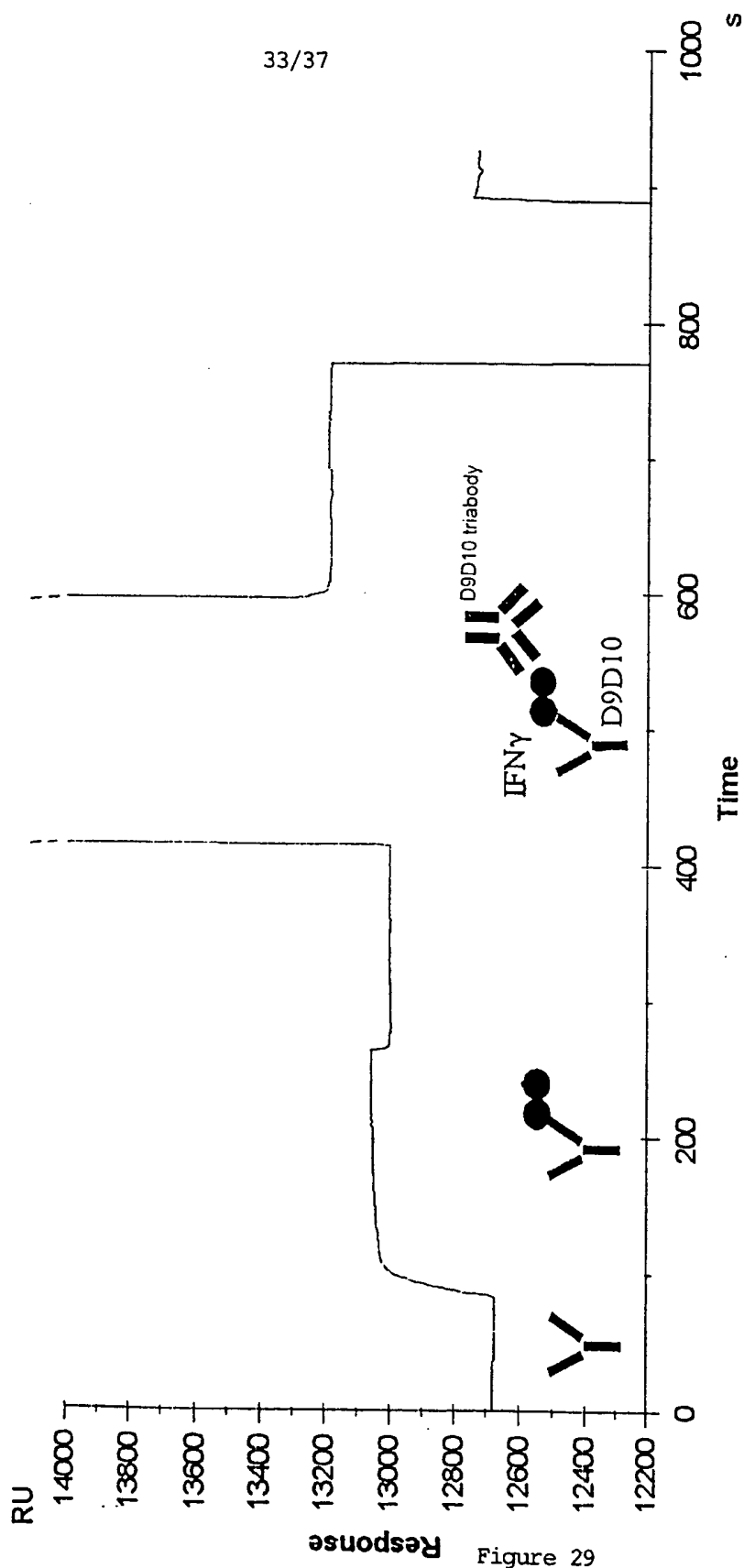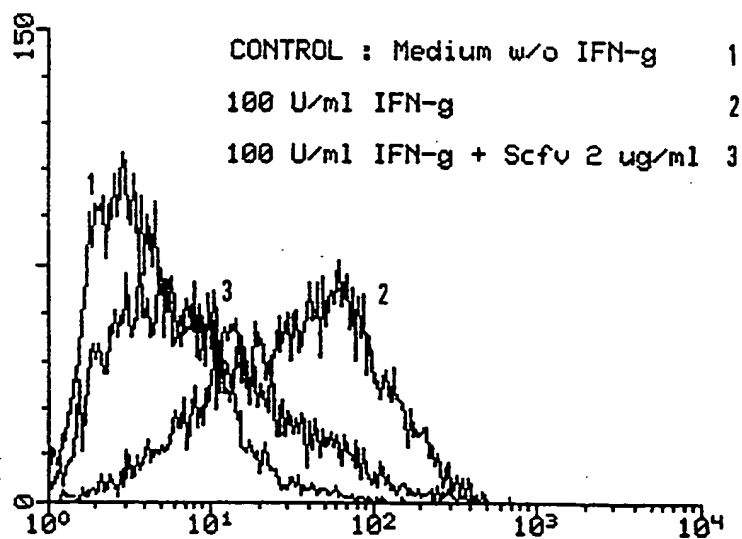
Figure 25

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  1    CAGGTGCAGCTGGTGCAGAGCGGTAGCGAACTGAAAAAACCGGGTGCGAG
 51    CGTTAAGATCAGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
101    TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGG
151    ATCAACACCTACACCGGTGAAAGCACCTACGTTGACGATTTCAAAGGTCG
201    TTTCGTTTTCAGCCTGGATACCAGCGTTAGCGCGGCCTACCTGCAGATCA
251    GCTCTCTGAAAGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
301    TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTC
351    AGGCGGTGGCGGATCGGACATCGTACTGACCCAGAGCCCGGCGACCATGA
401    GCGCGAGCCCGGGTGAACGTGTTACCCTGACCTGCAGCGCGAGCTCTAGC
451    ATCAGCTATATGTTCTGGTATCATCAGCGTCCGGGTCAGAGCCCGCGTCT
501    GTTGATCTATGATACCAGCAACCTGGCGAGCGGTGTTCCGGCGCGTTTCA
551    GCGGTAGCGGTAGCGGTACCAGCTATAGCCTGACCATCAGCCGTATGGAA
601    CCGGAAGATTTCGCGACCTATTTCTGCCATCAGAGCTCTAGCTATCCGTT
651    CACCTTCGGTCAGGGTACCAAACTCGAGATCAAACGG
```

Figure 26

Figure 27

```
1    CAGGTGCAGCTGGTGCAGAGCGGTAGCGAACTGAAAAAACCGGGTGCGAG
51   CGTTAAGATCAGCTGCAAAGCGAGCGGTTATACCTTCACCGATTACGGTA
101  TGAACTGGGTTAAACAGGCGCCGGGTCAAGGTCTGAAATGGATGGGTTGG
151  ATCAACACCTACACCGGTGAAAGCACCTACGTTGACGATTTCAAAGGTCG
201  TTTCGTTTTCAGCCTGGATACCAGCGTTAGCGCGGCCTACCTGCAGATCA
251  GCTCTCTGAAAGCGGAAGACACCGCGACCTACTTCTGCGCGCGTCGCGGT
301  TTCTACGCGATGGATTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTC
351  AGACATCGTACTGACCCAGAGCCCGGCGACCATGAGCGCGAGCCCGGGTG
401  AACGTGTTACCCTGACCTGCAGCGCGAGCTCTAGCATCAGCTATATGTTC
451  TGGTATCATCAGCGTCCGGGTCAGAGCCCGCGTCTGTTGATCTATGATAC
501  CAGCAACCTGGCGAGCGGTGTTCCGGCGCGTTTCAGCGGTAGCGGTAGCG
551  GTACCAGCTATAGCCTGACCATCAGCCGTATGGAACCGGAAGATTTCGCG
601  ACCTATTTCTGCCATCAGAGCTCTAGCTATCCGTTCACCTTCGGTCAGGG
651  TACCAAACTCGAGATCAAACGG
```

```
1   QVQLVQSGSELKKPGASVKISCKASGYTFTDYGMNWVKQAPGQGLKWMGW
51  INTYTGESTYVDDFKGRFVFSLDTSVSAAYLQISSLKAEDTATYFCARRG
101 FYAMDYWGQGTTVTVSSDIVLTQSPATMSASPGERVTLTCSASSSISYMF
151 WYHQRPGQSPRLLIYDTSNLASGVPARFSGSGSGTSYSLTISRMEPEDFA
201 TYFCHQSSSYPFTFGQGTKLEIKRHHHHHH
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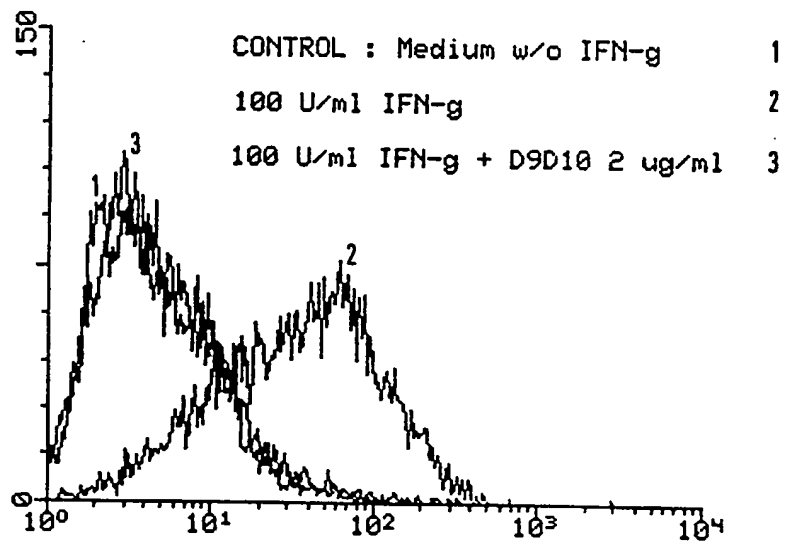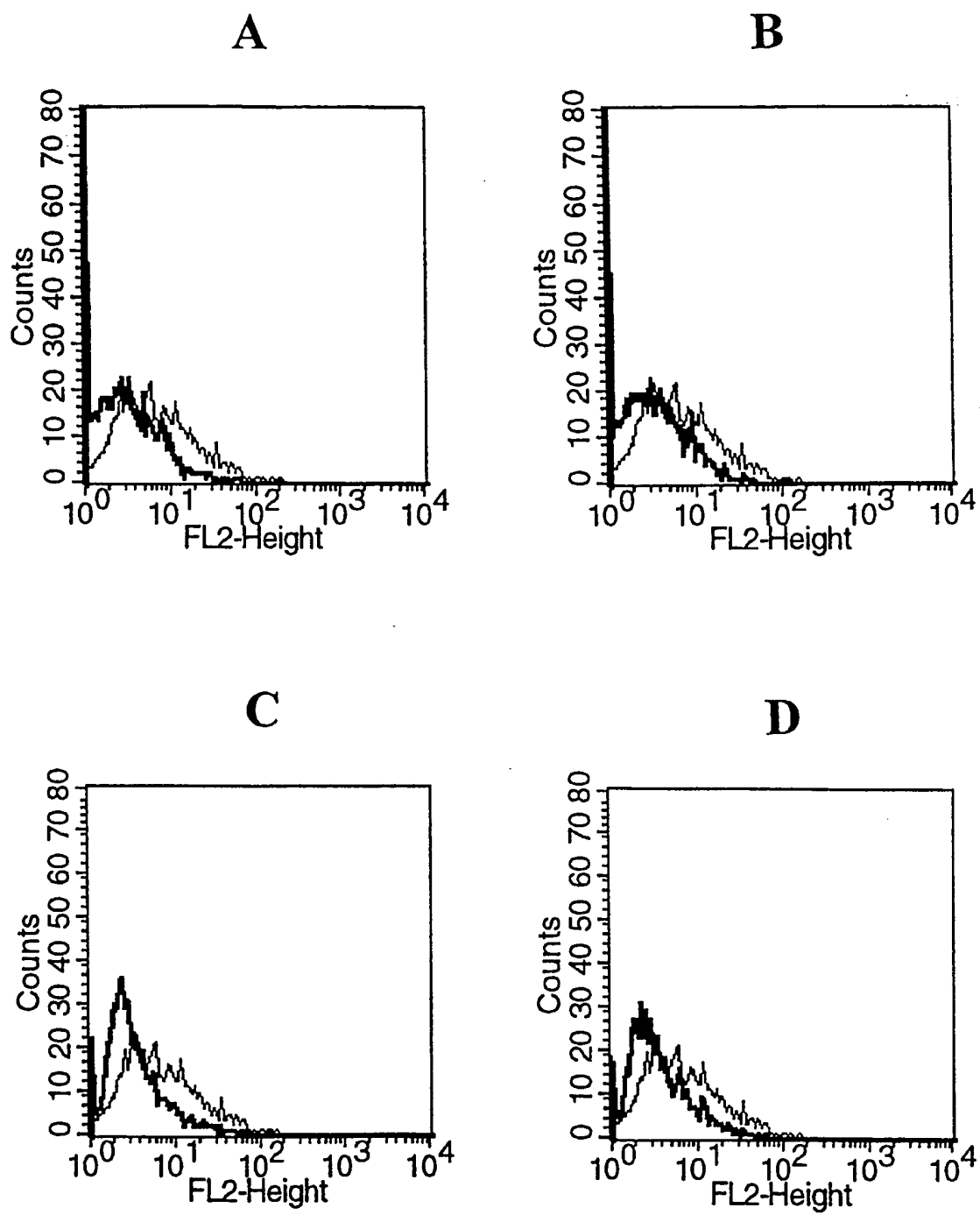
Figure 28

Figure 29

Figure 30
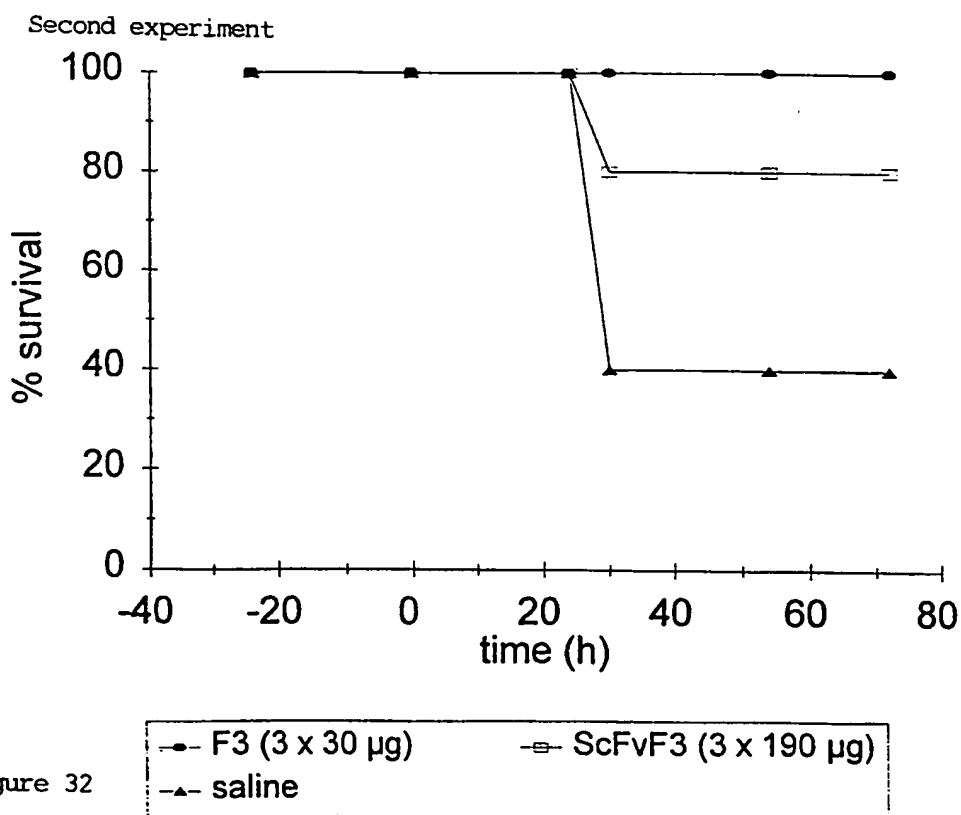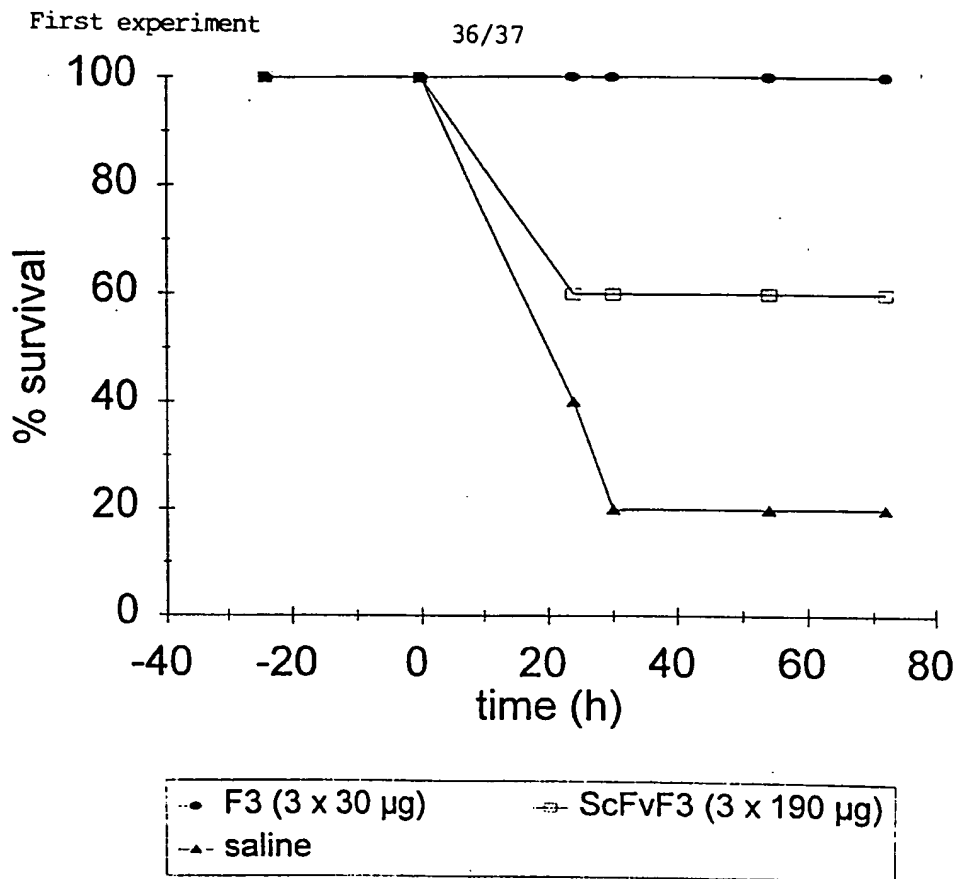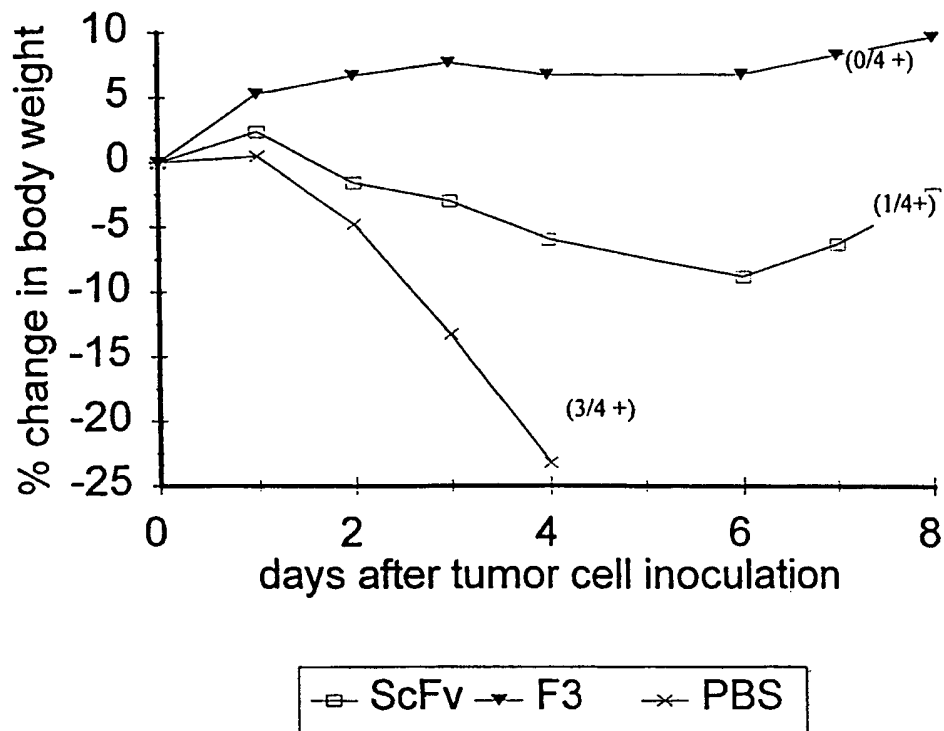
Figure 31

First experiment

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Second experiment

Figure 32

First experiment

Second expreriment

Figure 33